THE ECOTOXICOLOGY OF DIFFERENT FORMS OF COPPER (NANO, MICRO AND SALT) IN MARINE MUSSELS

By

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A thesis Submitted for the Degree of

Doctor of Philosophy

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October, 2015

ABSTRACT

Copper oxide nanoparticles (CuO NPs) are one example of NPs that are used in many applications, such as batteries, semiconductors and inks. The growing usage of CuO NPs will likely result in an increased release into the environment, especially the marine environment. Furthermore, there is a lack of understanding of the toxicity of CuO NPs on environmental species and humans, particularly marine benthic filter feeders where data are particularly lacking. Therefore, the focus of the current study was to assess the cytotoxicity (cell viability) and genotoxicity (DNA damage and oxidative stress) of different forms of Cu in relevant biomarker organisms, the blue mussels *Mytilus edulis* and the horse mussel *Modiolus modiolus.* Both mussels were exposed to different concentrations (5, 10, 15 and 20 μgL^{-1}) of different forms of particulate CuO (NPs, microparticles, MPs) and copper salt ($CuSO_4$), for 72 hours. Flow cytometry and trypan blue techniques were used to assess the viability of the haemocytes of the mussels and the Comet assay was used to assess DNA damage in the haemocytes and gill cells of the mussels. Superoxide dismutase (SOD assay) and the thiobarbituric acid reactive substances (TBARS assay) were used to assess oxidative stress measuring SOD enzyme activity and lipid peroxidation level in gill cells of the mussels after the exposure period (72 hours).

Results indicated that all three forms of Cu (NPs, MPs and salt) have the potential to decrease the cell viability in haemolymph cells for both type of mussels, *M. edulis* and *M. modiolus* in a concentration response manner. Similarly, NPs, MPs and salt forms of Cu caused DNA damage in both types of cells (haemolymph and gill) for *M. edulis* and *M. modiolus* mussels even at low concentrations ($5\mu gL^{-1}$), which is consistent with the cell viability results. Moreover, SOD activity and lipid peroxidation were observed to have increased in the cells of mussels exposed to all forms of Cu, indicating oxidative stress. Overall, the nano form of Cu appears to be more toxic to both mussel species, more than the micro and salt forms. These effects are primarily influenced by the special features of NPs, particularly their small size and large surface area, which affect the amount of Cu ions released into the exposure medium and inside the exposed cells. Furthermore, *M. edulis* mussels were more sensitive to Cu forms than *M. modiolus*, especially when exposed to nano forms of CuO. Furthermore, the use of both mussel species (*M. edulis* and *M. modiolus*) as bioindicator organisms is useful in the determination of the toxicity of the different Cu forms (nano, micro and salt).

Dedication

This thesis is dedicated to

My great father Mausa Alnashiri
His words of inspiration and encouragement
In pursuit of excellence and success

My lovely mother Lyla Alnashiri
With love and eternal appreciation, her measureless support, encouragement, and constant love have sustained me throughout my life

My wonderful wife, Fatmah
Whose was the love and support made throughout my period of study in UK

My beloved brothers and sisters
With love and support sustained me throughout my life

My beloved children, Hatan and Mausa
With love and being a great part of my success

ACKNOWLEDGMENTS

First of all, I will never be able to do anything in my life including this project without an enormous help and support of my God (Allah), who created me from nothing, raised me in peaceful environment, send prophets with guidance for the current and next lives, made everything possible to achieve by providing me a great ability to be successful and excellent.

I would like to express my very great appreciation and thank to my beloved parents, who have spent their lives looking after me, raised me with great attitudes, made everything available for me as much as possible with happiness and patience and supported me during the study with words of inspirations and encouragement in pursuit of excellence.

I would like to express my deep gratitude to Prof. Teresa Fernandes and Dr. Mark Hartl my research supervisors, who were always, supported me with enthusiastic encouragement, useful critiques and great advice throughout this study with patience, enabled me to improved my personal skills either in practical or theoretical understanding of the work. Nothing could be achieved in this project without their great and enormous support, guidance and advices, and I will appreciate it for the rest of my life.

I would like to show my gratitude to my sweetheart my wife Fatmah for her huge support and encouragement with love and happiness throughout the period of the study in UK. I also would like to thank my kids (Hatan and Mausa), my brothers and sisters and the all members of my family for their encouragement, interest and support during my studies, which enable me to concentrate on my project research without worrying by their advices.

This project could not have been completed without the further help and support of the kind colleagues, who helped and advised me during my work, Dr Majed Alshaeri, Dr Omar Alharbi, Dr Virginia Echavarri Bravo. In addition, I am also grateful to all the staff at the School of Life Sciences; especially to Dr John Kinross, Sean McMenamy, Hugh Barras, Paul Cyphus, Margaret Stobie, James Buchanan, Dr Dan Harries, Dr Bill

Sanderson, Dr Joana Porter, Clara Makenzie, Flora Kent, Robert Cook and the Nanosafety research group for their support, advice and assistance since the start of my project in 2012 and also during difficult times. Also, I'm deeply thankful to staff at University of Edinburgh Mr. Steve Mitchel, for his support in the TEM unit and Dr Lorna Eades for her support in chemical analysis at the School of Chemistry at Edinburgh University.

I would like to express my grateful thank to Jazan University for the scholarship and funding my study in UK. A special thanks to Saudi Arabian Cultural bureau in London for their advice and support during the period of my study. A special thanks to Dr Zarag Al-Faifi, who was supported me when I was studying Bachelor at King Abdul Aziz University as well as his help and encouragement since I worked at Jazan University until now. I owe my deepest gratitude to my beloved late friend Dr. Jamal Abo-Qura, who passed away in 2013, for encouraging and supporting me at work at Jazan University and study period in UK.

Finally, I would like to thank my friends for their big support and encouragement making my study easier away from my home and family. In particular I thank Ahmed Alsayed, Nawaf Mirza, Omar Mari, Mohammed Alomari, Azib Alqhanmi, Hamed Algarni, Sami Almalki, Faisal Alqahtani, Mohammad Algahtani, Atef Hobani, Abdullah Alzhrani, Yahia Alajlani, Ali Alshomrani, Metab Algamdi, Meshari Alesami, Ali Koam, Hamed Alyoubi, Majed Alsubih, Abdulaziz Rajab, Abdulazeem Alotabi, Meshal Abo Obead, Hamad Alhasson, Fahad Albelwi, Caroline Davis, and Jenifer Sponsor.

ACADEMIC REGISTRY

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Signature:		Date:	

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Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (In draft), The toxicity of particulate (nano and micro scale) and ionic forms of copper to the marine horse mussel (*Modiolus modiolus*), *Marine Environmental Research*.

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (In writing stage), the ecotoxicology of different forms of copper (nano and micro and salt) to the marine blue mussel (*Mytilus edulis*), *Marine Environmental Research.*

LIST OF CONFERENCE ORAL PRESENTATIONS

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2014). "Comparative toxicity of nano and microscale copper oxide particles in marine mussels", Society of Environmental Toxicology and Chemistry (SETAC) 24th Europe Annual Meeting in Basel, Switzerland.

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2014). "Comparative toxicity of nano and microscale copper oxide particles in marine mussels", Marine Alliance for Science and Technology for Scotland (MASTS) 4th Annual Meeting in Edinburgh, United Kingdom.

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2015). "The toxicity of particulate (nano and micro scale) and ionic forms of copper to different marine mussels (*Mytilus edulis and Modiolus modiolus*)", 10th Annual Postgraduate Research Conference, Heriot Watt University, Edinburgh, United Kingdom.

LIST OF CONFERENCE POSTERS

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2013). "The ecotoxicology of copper nanoparticles in marine mussels", Society of Environmental Toxicology and Chemistry (SETAC) 23rd Europe Annual Meeting in Glasgow, United Kingdom.

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2013). "The ecotoxicology of copper nanoparticles in marine mussels (*M. edulis*)", Marine Alliance for Science and Technology for Scotland (MASTS) 3rd Annual Meeting in Edinburgh, United Kingdom.

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2013). "The ecotoxicology of copper nanoparticles in marine mussels", 1st PhD Students Conference of School of Life Sciences at Heriot Watt University in Edinburgh, United Kingdom.

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2014). "DNA damage and oxidative stress in the gill cells of *M. modiolus* mussels exposed to different forms of copper oxide (NPs & MPs)", Society of Environmental Toxicology and Chemistry (SETAC) 24th Europe Annual Meeting in Basel, Switzerland.

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2014). "The genotoxicity of different forms of copper oxide (NPs & MPs) in *M. modiolus* mussels", Marine Alliance for Science and Technology for Scotland (MASTS) 4th Annual Meeting in Edinburgh, United Kingdom.

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2015). "The toxicity of particulate (nano and micro scale) and ionic forms of copper to the marine horse mussel (*Modiolus modiolus*)", Society of Environmental Toxicology and Chemistry (SETAC) 25th Europe Annual Meeting in Barcelona, Spain.

Alnashiri, H. M, Hartl, M. G. J and Fernandes, T. F. (2015"The toxicity of particulate (nano and micro scale) and ionic forms of copper to different marine mussels (*Mytilus edulis* and *Modiolus modiolus*)", Marine Alliance for Science and Technology for Scotland (MASTS) 5th Annual Meeting in Glasgow, United Kingdom.

LIST OF ABBREVIATIONS

AAS	Atomic absorption spectrometry
AFM	Atomic force microscope
Ag	Silver
Ag NPs	Silver nanoparticles
ALS	Alkali-labile sites
ANOVA	One-way analysis of variance
AP-1	Activator Protein-1
BCE	Before the Common/Current/Christian Era
BHT	Butylated hydroxytoluene
BPs	Bulk particles (*aka* microparticles; MPs)
BSA	Bovine Serum Albumin
CA	Chromosomal aberration assay
CAT	Catalase activity
Cl^-	Chlorides
CLSM	Confocal laser scanning microscope
CNTs	Carbon nanotubes
CO_2	Carbon dioxide
Cu	Copper
CuO NPs	Copper oxide nanoparticles
CuO MPs	Copper oxide microparticles

$CuSO_4$	Copper sulphate
Cu/Zn SOD	Copper zinc superoxide dismutase (SOD1)
CV	Crystal violet
DDH_2O	Double distilled water
DH_2O	Distilled water
DIW	Deionised water
DLS	Dynamic light scattering
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break
DSS	DNA single strand
EC-SOD	Extracellular superoxide dismutase SOD3
EDS	Energy dispersive X-ray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
EELS	Elemental analysis electron loss spectroscopy
ENMs	Engineered nanomaterials
ENPs	Engineered nanoparticles
ERKs	Extracellular-receptor kinases
EC	European Commission
FC	Flow cytometry
Fe_3O_4 and Fe_2O_3	Iron oxide

FSC	Forward scatter cytometry
GP_X	Glutathione peroxidases
GSH	Glutathione
GSSG	Oxidized glutathione
HBSS	Hanks buffered salt solution
HCl	Hydrogen chloride
HCMECs	Hepatocellular cardiac microvascular endothelial cells
HEp-2	Hepatic epithelial cells type 2
HepG2	Hepatocellular carcinoma cells type 2
HO^-	Hydroxyl radical
H_2O-	Hydroperoxyl anion
H_2O	Water molecular
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
ICP-OES	Inductively coupled plasma optical emission spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry
JNK/SAPK	Jun N-terminal kinase/stress activated protein kinase
KCl	Potassium chloride
LMP	Low melting point Agarose
MAPK	Mitogen-activated protein kinases
MDA	Malondialdehyde

MNT	Micronucleus test
MnSOD	Manganese superoxide dismutase (SOD2)
MPs	Micro particles
MT	Metallothioneins
MTT	Tetrazolium bromide
MWCNTs	Multi walled carbon nanotubes
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NGA	Normal Gel Agarose
NH_3	Ammonia
NHO_3	Nitric acid
NMs	Nanomaterials
NO^-	Nitric oxide
NPs	Nanoparticles
NR	Neutral red
NRA	Neutral red assay
NSMs	Nanostructured materials
NSOM	Near-field scanning optical microscope
O_2^-	Superoxide radicals
PBS	Phosphate buffered saline
Pd	Palladium

Pi	Propidium iodide
PO_4^{3-}	Phosphates
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
ROO	Peroxyl radical
SAED	Selected area electron diffraction
SCE	Sister-chromatid exchange assay
SCGE	Single cell gel electrophoresis
SEM	Scanning electron microscope
SOD	Superoxide dismutase
SSA	Specific surface area
SSC	Side scatter cytometry
SWCNTs	Single wall carbon nanotubes
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEM	Transmission electron microscope
TiO_2 NPs	Titanium oxide nanoparticles
Tris	Tris hydrochloride
WST	Working solution
ZnO NPs	Zinc oxide nanoparticles

1 INTRODUCTION

This chapter provides a brief overview of the experimental chemicals selected for use in this research, beginning with the elemental form of copper (Cu). It then introduces nanomaterials (NMs); explaining what they are, their sources, their physical and chemical properties, their applications, and finally their fate and ultimate impact on the environment. After this an in depth description of copper oxide nanoparticles (CuO NPs) as a nano form of Cu metal will be reviewed in reference to the use of CuO NPs, their fate, including an outline of related literature concerning the toxicity of CuO NPs to living organisms, particularly aquatic mussels. Following this, the selection of the experimental organisms (*Mytilus edilus* and *Modiolus modiolus*) will be discussed, in particular the reasons for choosing these specific species. This will be followed by a description of the biomarkers and research methods used in the study; including cell viability techniques such as flow cytometry (FC) and trypan blue, single cell gel electrophoresis or Comet assay, superoxide dismutase (SOD) assay, and thiobarbituric acid reactive substances (TBARS) assay. Furthermore, this chapter also covers the characterisation and accumulation analysis techniques for all forms of Cu, i.e. transmission electron microscope (TEM), dynamic light scattering (DLS) and inductively coupled plasma optical emission spectrometry (ICP-OEM). Finally, the aims of this study are stated and the specific research objectives and related questions given.

1.1 Elemental Copper (Cu)

Copper (Cu) belongs to group 11 of the periodic table, as do the elements silver and gold, with which it shares some properties, including the occurrence of colour and high electrical and thermal conductivity (Emsley, 2001; Ayres et al., 2002). Cu as some other metals occurs in the crust of the earth (c. 7000 BCE in Turkey). Cu was used for various purposes, and it was the second metal that used by human in many applications mainly for decorative purposes (Ayres et al., 2002). Cu atoms are presented on Earth as two naturally occurring isotopes, ^{63}Cu (70%) and ^{65}Cu (30%). Cu has two common oxidation states, Cu^{+} and Cu^{2+} involved in the formation of most Cu compounds (Ayres et al., 2002).

The distribution of Cu and its forms in fresh water depends on the pH and alkalinity of the water, as well as the presence of inorganic compounds, such as hydrogen sulphide (H_2S), phosphates (PO_4^{3-}), chlorides (Cl^-) and ammonia (NH_3) (Ayres et al., 2002). Cu is an active element, because of its tendency to interact with organic and inorganic materials containing nitrogen or sulphur to form strong chemical compounds in water or soil. In addition, Cu can also form complexes with large macromolecules, such as proteins and nucleotides (Ayres et al., 2002).

Cu is an important element found in small quantities in the cells and tissues of all species, with highest concentrations in the liver (Turnlund 1998). Cu is an essential component in over 10 enzymes, contributing to their structural and catalytic properties (e.g. cytochrome c oxidase, lysyl oxidase, p-hydroxyphenyl pyruvate hydrolase, dopamine beta hydroxylase). These enzymes are important actors in biological processes including growth, development and help to resist diseases (Emsley 2001). Other important enzymes dependent on Cu are superoxide dismutase (SOD), which protects against free radicals, and tyrosinase, which is essential to the pigment melanin (Gaetke et al., 2003).

Although Cu is essential to all living organisms, it can be toxic when present in high concentrations (it may reach 3 ppm in heavily polluted areas) or if organisms are exposed chronically to low levels of Cu in the environment (Gaetke and Chow 2003). According to Emsley (2001), “a child who ate the sample of Cu sulphate in a toy chemistry set died as a result”. The early symptoms of Cu poisoning after swallowing are vomiting; this reaction is the body’s way of trying to prevent acute toxicity (Emsley 2001). In addition, Ayres et al. (2002) reported that Cu compounds containing organic matter or Cu oxides are also unsuitable for ingestion by living organisms, due to their potential toxicity at certain concentrations. Researchers have repeatedly studied the effects of Cu on aquatic organisms. For example, researchers have shown that chronic exposure to Cu can cause impairment to the feeding mechanism of the Mytilidae mussel *Perna viridis* L, reduced growth rates, oxidative damage to lipids, proteins, and DNA, and contributes to neurodegenerative disorders and reproduction in the blue mussel (*Mytilus trossulus*) (Gaetke et al., 2003; Zorita et al., 2006; Al-Subiai et al., 2011).

Certainly, the toxicity of Cu and its bioaccumulation in aquatic organisms have been studied in detail, and can be relatively well understood from earlier studies. However, to the author's knowledge there has to date been no study assessing the toxicity of three forms of Cu on the same aquatic organisms. Since Cu is an important metal involved in many biological systems in a variety of organic compounds, the assessment of the uptake of different forms of Cu in aquatic organisms and its toxicity is an important area of concern.

1.2 Nanomaterials (NMs)

There remains much debate regarding the formal definition of nanomaterials (NMs), however the most commonly agreed definition of the NMs is that adapted by the European Commission (2011):

"Nanomaterial" means a natural, incidental or manufactured material containing particles (i.e. minute pieces of matter with defined physical boundaries), in an unbound state or as an aggregate (i.e. particles comprising of strongly bound or fused particles) or as an agglomerate (i.e. collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components) and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm. In specific cases, and where warranted by concerns for the environment, health, safety or competitiveness, the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %" (European Commission (2011/696/EU)).

This definition conveys the fact that nanomaterials can be one dimensional particles (nanofilms and nanosheets), two dimensional particles (nanowires and nanotubes), or three-dimensional particles of almost any shape; together they are commonly described as nanoparticles (NPs) (Handy et al., 2008; Oberdörster 2009; Bernhardt et al., 2010; Klaine et al., 2012).

There are two main sources of NMs: natural and unnatural (also known as anthropogenic, both incidental and engineered). Natural nanomaterials are abundant and arise from biological or geological mechanisms that have occurred during the natural functioning of the ecosystem since the formation of the earth; for example, NMs have been found in glacial ice cores as old as 10,000 years (Murr et al. 2004; Bhatt and Tripathi 2011). Sources of natural NMs include volcanic eruptions, simple erosion, sea salt aerosols, photochemical reactions, forest fires, and other transition metal oxides in soils, rivers, and oceans (Bernhardt et al., 2010; Blinova et al., 2010; Casals et al., 2012). The natural NMs present in the environment play an important role in biogeochemical processes for example, the atmosphere contains small particles suspended in the air (known as aerosols), which balance the energy received from the sun by distributing some of it back in to space (Buzea et al., 2007; Bhatt and Tripathi 2011).

The other source of NMs is anthropogenic or human activities, which can result in the release of either incidental or manufactured (also referred to as engineered) NMs. Incidental NMs include materials produced unintentionally or as by-products of human activities such as welding, transport, refining, smelting, chemical manufacturing, energy production by combustion of sewage sludge, coal and fuel oil (these NMs include carbon black, carbon nanotubes and fullerenes, platinum, and rhodium) (Buzea et al., 2007; Dhawan and Sharma 2010). Engineered or manufactured NMs are produced for their unique physical and chemical properties. These NMs are key ingredients in many commercial products (e.g. toothpaste, tyres, sunscreen and stain-resistant clothing) (Buzea et al., 2007; Casals et al., 2012).

Natural and manufactured NMs have valuable, unique physical, chemical and biological properties (Figure 1.1). Typically, their utility results from their small size, large surface area and homogeneous composition. For example, NMs have a larger specific surface area (SSA) than their larger counterparts comprised of the same materials, and the proportion of atoms on the surface versus the interior of the particle is also much larger for NPs which may lead to an increase in the surface reactivity of these materials (such as adsorption and catalytic properties) (Handy et al., 2008; Lövestam et al., 2010). Thus, the surface area of these materials is an important factor in their toxicity which linked to the particles size, since biological and chemical reactions often take place at their

surfaces, one expects nanomaterials to be much more reactive than the same mass of material made up of coarser structures (Dhawan et al 2010 and Lovestam et al., 2010). For example, in the spherical shaped nanoparticles, the specific surface area increases with the inverse of the diameter. From purely geometrical considerations, as an example, 10g of silver in the form of spherical nanoparticles with a diameter of 10nm exhibits a total surface area of almost 600cm^2. This should be compared to a single solid silver sphere with the same mass which has a surface area of nearly 5cm^2, giving an increase in total surface area for the nanomaterial form of a factor of about 1,200,000 (Lovestam et al., 2010). The properties of these NMs commonly manifest differently as a consequence of several factors, including physical factors (such as size), biological factors (pH and salinity) and the change in NMs behaviour in different media (Oberdörster 2009; Bernhardt et al., 2010).

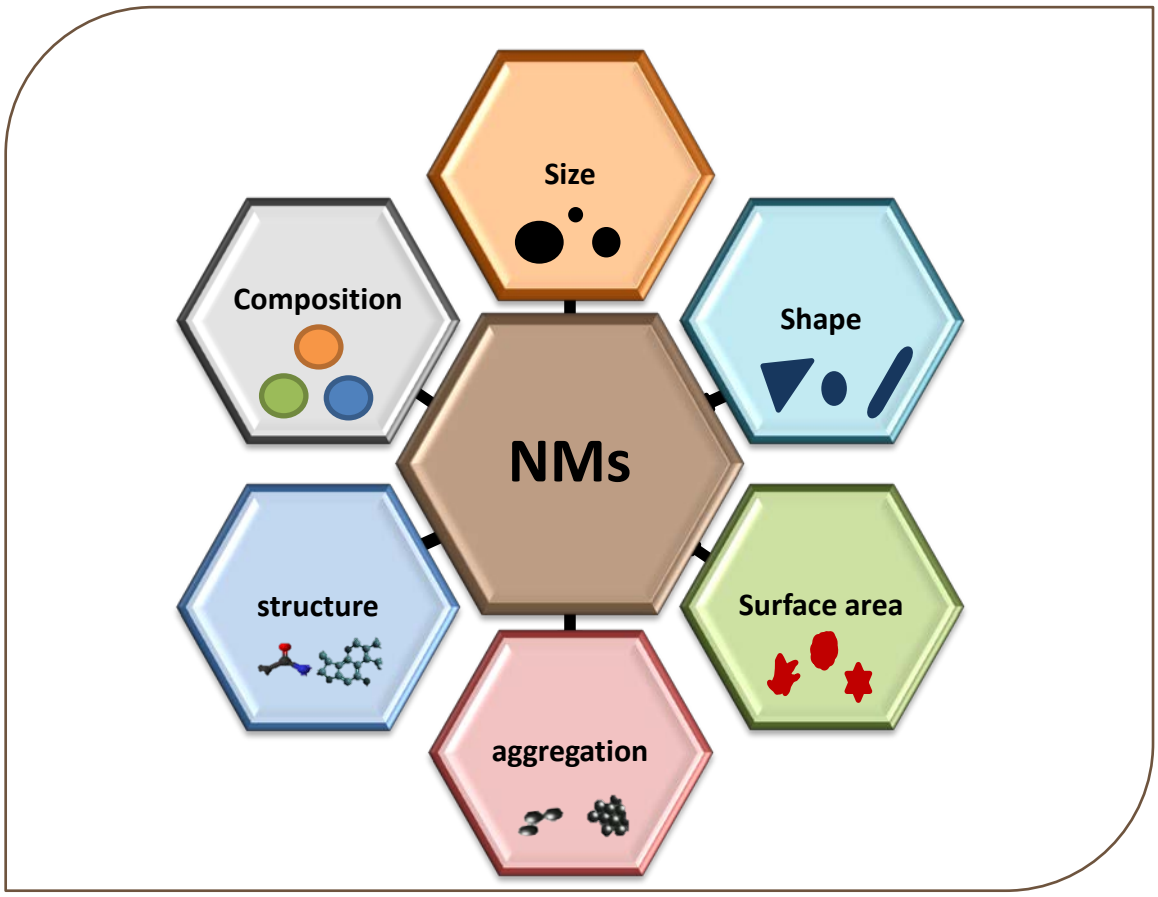


Figure 1.1: The main physicochemical properties for NMs and their interrelation

The size of the NM is the most essential and critical parameter, because of its influence on other properties, including physical (surface area and zeta potential), chemical (reactivity and solubility, conductivity and redox behaviour) and mechanical (elasticity and hardness) (Oberdörster 2009; Dhawan and Sharma 2010). Additionally, NMs can be easily introduced to biological systems such as the human body because of their small size. They can cross the biological barriers in the lung, gut, or the brain, and the aggregation of NMs inside the biological system can alter biological responses due to a reduction in the surface charge (Lövestam et al., 2010; Bhatt and Tripathi 2011). Surface charge is another important parameter that can influence the behaviour and the toxicity of NMs in living organisms, since the interaction of these particles with biological systems occurs on their surfaces (Dhawan and Sharma 2010; Casals et al., 2012). Several comprehensive studies have extensively discussed NMs and their properties, and how these particles may impact the environment (Buzea et al., 2007; Handy et al., 2007, 2008 and 2011; Klaine et al., 2008 and 2012; Oberdörster 2009; Bernhardt et al., 2010; Peralta-Videa et al., 2011; Bhatt and Tripathi 2011).

The unique physiochemical properties of NMs, such as large surface area and small size have resulted in considerable use of NMs in commercial and industrial applications and products in daily use. These properties can improve products by increasing the quality of life through more efficient target driven pharmaceuticals, better diagnostic tools for medical diagnosis, faster computers, and cleaner energy production (Bystrzejewska-Piotrowska 2009; Lövestam et al., 2010). The large number of applications containing NMs employed in medical and healthcare products, consumer electronics, easy-clean surface coatings, catalysts and lubricants, paints and coatings, construction materials, sports equipments, cosmetics and personal care products, household products, textiles, food and nutritional ingredients, as well as weapons and explosives (Lövestam et al., 2010; Hartmann 2010; Bhatt and Tripathi 2011).

Investigating the potential impact of NMs on the environment is important because these materials are becoming pervasive in our society and their use is likely to continue to increase in the near future. A concern has been raised regarding the impacts of these particles on the environment, especially on living organisms including humans (Bondarenko et al., 2013). A vast body of literature is available detailing studies of the effects and toxicity of manufactured NMs on humans and other living organisms, such

as Moore (2006); Oberdörster et al. (2006 and 2009); Crane and Handy (2007) and Handy et al. (2008 and 2011) (See pages 11-16 for CuO NPs toxicity).

It has been suggested that not all engineered NMs impact health, and the toxicity of NMs is believed to be influenced by several factors, such as composition, shape, surface functionality, crystallinity, aggregation and size (Figure 1.1) (Buzea et al., 2007; Bystrzejewska-Piotrowska et al., 2009). In addition, prediction of the effects of NMs and NPs on the environment requires studies of several factors that may alter the behaviour of the NPs and therefore their toxicity such as:

- The fate, the form and mass of engineered NMs entering the environment;
- The behaviour of NMs in environmental media;
- The reaction of living organisms to exposure to the NMs; and
- The effects of NMs input on ecological communities and biogeochemical processes (Handy et al., 2008; Bernhardt, et al., 2010).

NMs can enter the human body in three ways: through the skin, the lungs, and the gastro-intestinal tract, since all these are in contact with the surrounding environment. Example of effects on human health attributable to NMs include diseases associated with inhaled NMs such as lung cancer, asthma, emphysema, bronchitis and neurodegenerative diseases, such as Parkinson's (Buzea et al., 2007; Bystrzejewska-Piotrowska et al., 2009). Furthermore, exposure to silver NPs (Ag NPs) leads to oxidative stress, calcium transience, cytotoxicity and chromosome instability, intracellular and cell cycle arrest in many aquatic organisms (Bar-Ilan et al., 2009; Wise Sr et al., 2010). Additionally, metal oxides such as zinc oxide (ZnO), titanium oxide (TiO_2) and CuO NPs have diverse effects on aquatic organisms (e.g. fish, *Daphnia magna* and *zebrafish*). These impacts include DNA damage, cytotoxicity, apoptosis and inflammatory response, alteration of calcium homeostasis, oxidative stress, membrane damage and lysosomal membrane destabilisation (Gaiser et al., 2009; Jingxia et al., 2009; Dhawan and Sharma 2010).

1.2.1 Entry and fate of Nanomaterials in Aquatic Environments

NMs enter the environment from two main sources, i.e. intentional releases and unintentional releases (Figure 1.2). An example of the intentional release of NMs includes their use in personal healthcare products (sunscreens and cosmetics), paints and fabrics. In contrast, unintentional sources of NMs include atmospheric emissions and solid or liquid waste streams that generate from production facilities (Klaine et al., 2008; Casals et al., 2012). Furthermore, in some cases a high volume of NMs might enter the environmental because of spillage during the transportation of NMs between different manufacturing sites (Bhatt and Tripathi 2011; Klaine et al., 2012).

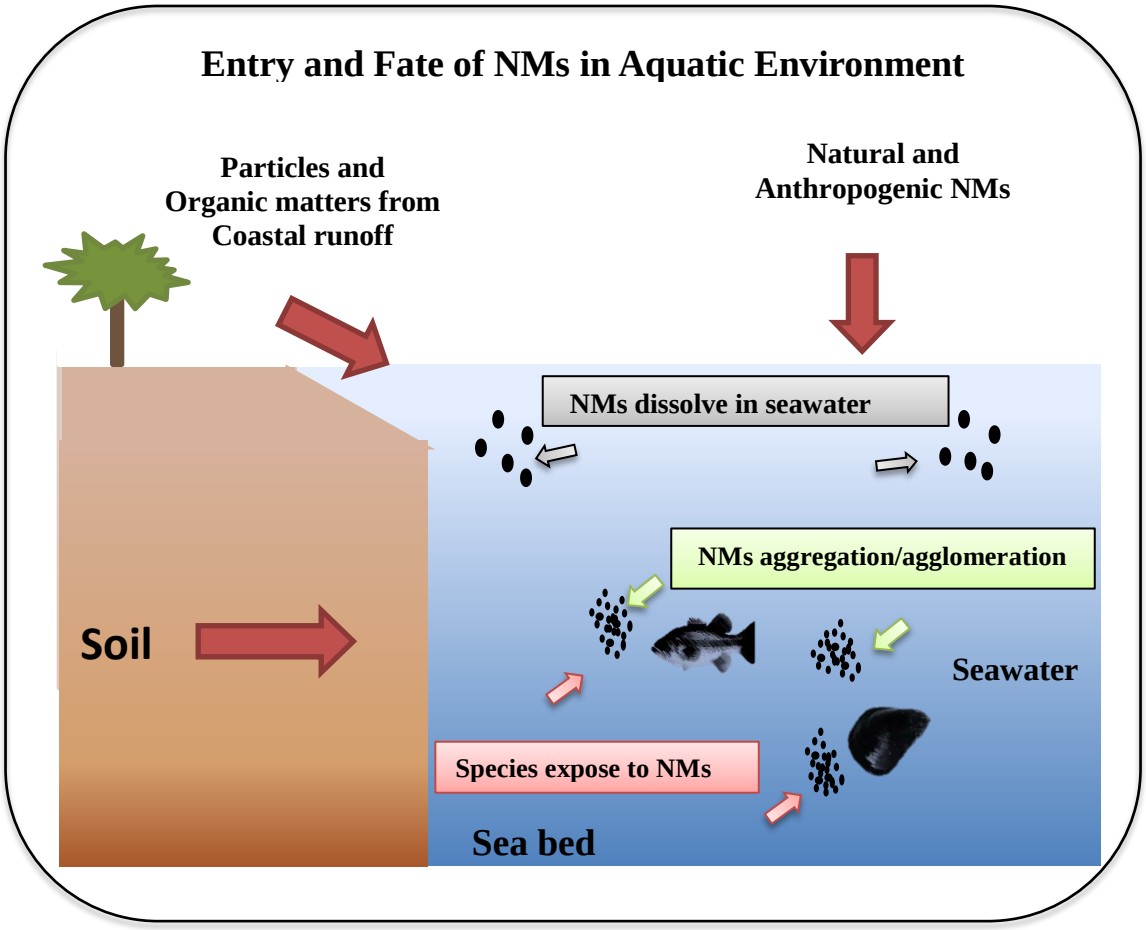


Figure 1.2: Potential fate of natural and engineered NMs released into the aquatic environment and how species may be exposed to NMs (adapted from Gaiser et al., 2011).

The aquatic environment is the receptacle of a variety of contaminants, including natural and engineered NMs from different sources as mentioned above. The presence of NMs in coastal regions might be a result of erosion of the coastal area, direct discharges, wastewater effluents, atmospheric deposition and river runoff. The amount of these NMs will increase gradually in the water column, and then precipitate to the substratum, where they can then interact with marine species that live at the bottom of the water column such as mussels (Klaine, et al., 2008; Casals et al., 2012). The fate of NMs in the aquatic environment depends on multiple factors, such as the existence of natural organic matter (NOM), ionic strength and pH, and the amount of NMs deposited into the aquatic environment is subject to variation (Rosse and Loizeau 2003; Bhatt and Tripathi 2011; Casals et al., 2012).

Despite the high number of publications related to NMs, very few data are available describing the fate and behaviour of engineered NMs in the environment. The detection of manufactured carbon-based NM concentrations is complicated due to several factors such as, low concentrations of engineered NMs and high concentrations of natural NMs such as colloids and organic carbon (Handy et al., 2008 and 2011; Bhatt and Tripathi 2011; Casals et al., 2012). The same applies to nanoscale forms of metals which are widely available in the environment, such as zinc. Furthermore, an estimation of the environmental concentrations of manufactured NPs demands more comprehensive understanding of the fate, transport and behaviour of manufactured NMs in the environment (Gottschalk et al., 2009; Klaine et al., 2012).

1.3 Copper Oxide Nanoparticles (CuO NPs)

Metal and metal oxide nanoparticles (such as Ag, Cu, Pd, ZnO, TiO_2 and CuO) are commonly utilized in large scale in various applications and products due to their unique electrical, magnetic and optical properties and also their chemical resistance, hardness and thermal stability (Mortimer et al., 2010; Dey et al., 2012). Examples of their applications include semiconductors, energy conversion and storage devices, microelectronics, fillers, biomedical applications, coatings, photonics, paints, cosmetics and catalysts (Anyaogu et al., 2008; Kahru et al. 2008; Blinova et al., 2010). Among these valuable transition metal oxide NPs, copper oxide has attracted the imagination of researchers, because of their typical structural lineaments (has a square planar

coordination of the Cu atom to the neighbouring oxygen atoms and a monoclinic crystal structure) and also broad range of existing and potential applications (Dey et al., 2012).

Copper oxide nanoparticles (CuO NPs) are increasingly used in a wide range of applications, such as gas sensors, semiconductors, photovoltaic cells, pigments in ceramics, optical equipment, microelectronics, cosmetics and catalytic processes (Ahamed et al., 2010; Dasari et al., 2013; Bondarenko et al., 2013), as coatings on integrated circuits, in inks and batteries, hospital equipments, antifouling paints, fabrication of solar cells, as bactericides in wood preservatives and for air and liquid filtration materials (

Figure 1.3) (Griffitt et al., 2007; Aruoja et al., 2009; Mortimer et al., 2010; Chibber et al., 2013). This widespread usage means that these nanoparticles will eventually lead to an increase of their presence in the air, soil and especially aquatic environments and may affect the organisms living therein (Dasari et al., 2013).

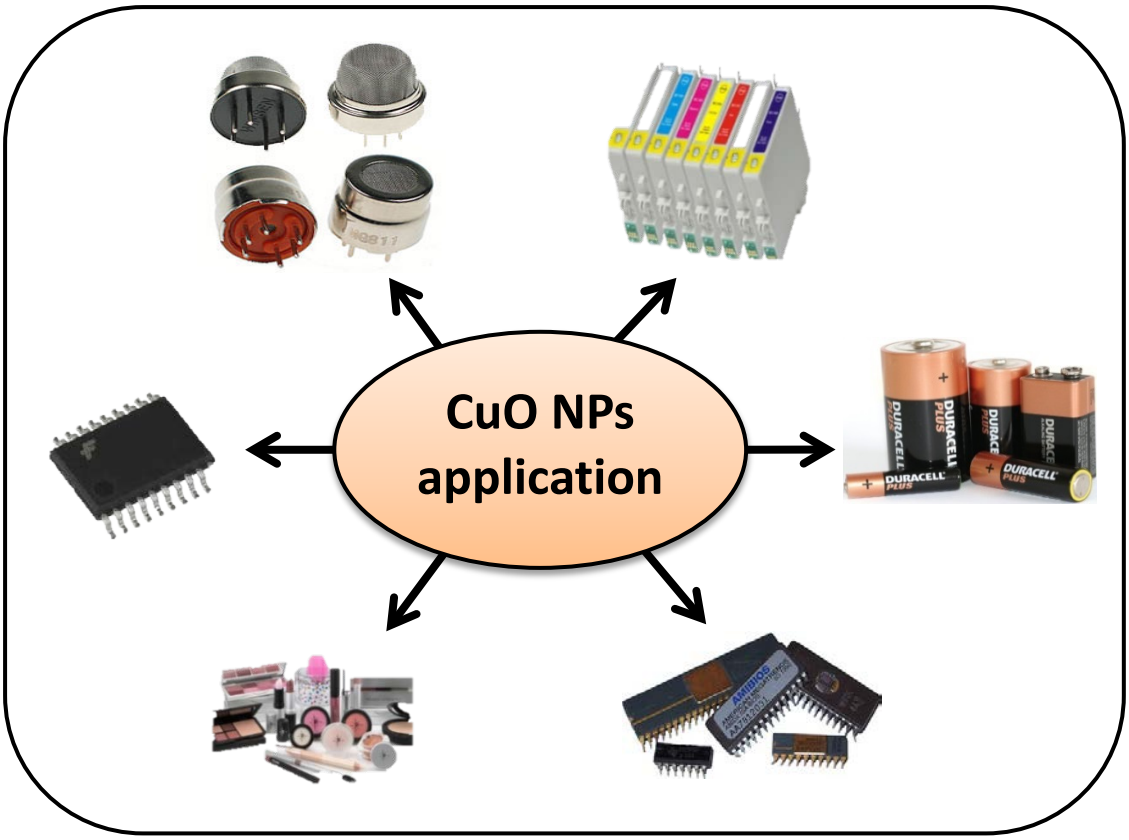


Figure 1.3: Examples of current applications of CuO NPs.

As explained earlier, a clear understanding of the fate of CuO NPs in the environment is still lacking, and no precise data are available to address this issue, as well as information on realistic environmental concentrations (Bhatt and Tripathi 2011; Casals et al., 2012; Bondarenko et al., 2013). CuO NPs will likely follow the same fate of other engineered nanoparticles such as Ag, ZnO and TiO_2 NPs, entering the aquatic environment through a variety of pathways, including wastewater effluents, landfill leakage waste and spillages during manufacturing and transportation, where they may accumulate, particularly in sediments and interact with the living biota (Figure 1.2) (Kahru et al., 2008; Buffet et al., 2011; Casals et al., 2012).

Numerous studies have evaluated the toxicity of CuO NPs in diverse organisms, including human, microorganisms and aquatic organisms. These studies have assessed a wide range of endpoints, for example oxidative stress, lipid peroxidation and cell viability. The following section detail existing literature concerning the effect of CuO NPs toxicity on mammalian cells in vitro, including human and mice cells. This will be followed by the CuO NPs toxicity on other organisms including bacteria, yeast, algae, and zebrafish. Finally, the toxicity of CuO NPs on mussel species will be explained separately as they the test organisms used in the present study.

1.3.1 Toxicity of CuO NPs in Mammalian cells

Several studies have investigated the cytotoxicity and genotoxicity of CuO NPs on human cells. Karlsson et al. (2008) assessed and collated findings relating to the toxicity of multiple metal oxide nanoparticles (including CuO NPs) (40 and 80μgmL^{-1} for 18 hours), carbon nanotubes (both single and multiwall) on the epithelial cell lining of human lungs (A549). The study reported that CuO NPs caused the most significant DNA damage (41% tail) following 4 hours exposure to 80μgmL^{-1}, decreasing cell viability by 93%. Subsequently, Karlsson et al (2009) conducted an additional study to compare the toxicity of nano and micro particles of some metal oxides (Fe_2O_3, Fe_3O_4, TiO_2 and CuO) on the human pulmonary epithelial cell line A549. The study showed CuO NPs were significantly more toxic than CuO MPs.

Fahmy and Cormier (2009) also investigated the toxic effects of multiple metal oxide NPs (SiO_2, Fe_2O_3 and CuO) on hepatic epithelial cells (HEp-2). Their study demonstrated that CuO NPs caused oxidative stress in a concentration dependent manner, resulting in cell death. Similarly, Ahamed et al. (2010) assessed the influence of different concentrations of CuO NPs (50nm) (10, 15 and 50μgmL^{-1}) on human pulmonary epithelial cells (A549), focusing on cytotoxicity and oxidative stress. Their results were in agreement with the previous studies mentioned above, revealing a reduction in cell viability following CuO NPs exposure. In addition, oxidative stress was observed following CuO NPs exposure in a concentration dependent manner manifested by the depletion of glutathione and induction of lipid peroxidation, catalase and superoxide dismutase. Ahamed et al. (2010) concluded that CuO NPs were genotoxic to human pulmonary epithelial cells (A549) and caused an increase in oxidative stress and lipid peroxidation.

In 2012, Wang et al. (2012) investigated the toxicity of CuO NPs (10−100mgL^{-1}) to human pulmonary epithelial cells (A549). They compared their toxicity with that of larger particles from the same compound (CuO bulk particles (BPs)). The results revealed that the larger particles resulted in less toxicity than the NPs (24 hours IC_{50}, 58 and 15mgL^{-1} for CuO bulk and NPs, respectively), and that CuO NPs also induced mitochondrial depolarisation, possibly mediated by the generation of reactive oxygen species (ROS). Piret et al. (2012) conducted experiments into the possible toxicity of two different forms of CuO (bulk and NPs) on human hepatocellular carcinoma cells (HepG2). They found that CuO NPs induce cellular toxicity by generating reactive oxygen species, also inducing activation of mitogen-activated protein kinases pathways (MAPK), phosphorylated extracellular-receptor kinases (ERKs) and phosphorylated c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK), which plays a major role in the activation of Activator Protein-1 (AP-1).

Siddiqui et al. (2013) conducted a similar study examining the possible mechanisms of apoptosis by exposing human hepatocellular carcinoma cells (HepG2) to CuO NPs (22nm) in a concentration range of 2–50$mgmL^{-1}$. This study showed that CuO NPs (concentration range 2–50 $mgmL^{-1}$) induced cytotoxicity HepG2 cells in a concentration dependent manner and that the results expressed the underlying mechanisms of

apoptosis was caused by CuO NPs exposure. It also indicated a need for further investigation at the *in vivo* level (Siddiqui et al., 2013).

1.3.2 Toxicity of CuO NPs in other organisms

Heinlaan et al. (2008) conducted a study to investigate the relative ecotoxicity of three different metal oxide NPs (ZnO, TiO_2 and CuO), and their larger forms on three different species: a bacterium (*Vibrio fischeri)* and two crustacean species (*Daphnia magna and Thamnocephalus platyurus*) in terms of measuring the half maximal effective concentration (EC_{50}) and growth inhibition of *V. fischeri* and mortality rate of *D. magna* and *T. platyurus*. The results of exposure to Cu compounds were that all three organisms experienced effects at all the concentrations tested (Heinlaan et al., 2008). These findings were similar to conclusions drawn by Hai-zhou et al. (2012), who indicated that a CuO and ZnO NPs mixture was the most toxic to *Daphnia magna* during the test period (48 hours).

Analogously, Mortimer et al. (2008, 2010 and 2011) conducted several studies assessing the genotoxic effects of two metal oxide NPs (CuO and ZnO) on different species, including bacteria (*Vibrio fischeri*) and protozoa (*Tetrahymena thermophile*); they also compared CuO NPs toxicity to larger form of CuO (bulk form). The outcomes of all the studies demonstrated that CuO NPs induced the generation of ROS, and were about 10–20 times more toxic than bulk CuO, although they were less toxic than ZnO NPs. Ivast et al. (2010) reported that CuO NPs generated higher level of ROS in the bacterium *E. coli*, than other metal oxide NPs (e.g. TiO_2 and ZnO).

Similarly, Dasari et al. (2013) also used *E. coli* to measure the cytotoxicity of several metal oxide NPs (ZnO, CuO, Co_3O_4 and TiO_2) in both light and dark conditions, and demonstrated that CuO NPs were the second highest toxic NPs to *E. coli*, under both conditions. Dimkpa et al. (2011) used pathogenic bacteria (*Pseudomonas chlororaphis* O6) to study and compare the toxicity of CuO and ZnO NPs. This study reported that CuO NPs were more toxic than the ZnO NPs, which disagreed with Mortimer et al.'s (2008 and 2011) results, mentioned above. Numerous other studies using bacteria species have observed the same toxic effects of CuO NPs (Yoon et al., 2007; Baek and

An 2011; Bondarenko et al., 2012; Pandey et al., 2012; Khan et al., 2013; Lu et al., 2013; Zhao et al., 2013; Wahab et al., 2013).

Other studies used several aquatic organisms to assess the CuO NPs toxicity. Aruoja et al. (2009) evaluated the toxicity of ZnO, TiO_2 and CuO NPs (TiO_2: 640mgL^{-1}; ZnO: 10mgL^{-1}; CuO: 100mgL^{-1}) on algae *Pseudokirchneriella subcapitata*. Their results indicated that CuO NPs were more soluble, and more toxic, than bulk CuO, and that CuO NPs were less toxic that ZnO NPs and more toxic than TiO_2 NPs. A comparative study was carried out by Pang et al. (2012), assessing the effect of CuO micro and nanoparticles deposited in sediments, on a deposit-feeding snail (*Potamopyrgus antipodarum*), in terms of their mortality, specific growth rate, feeding rate, reproduction, and bioaccumulation after 8 weeks of exposure to nominal concentrations of 0, 30, 60, 120 and 240µg Cu g^{-1} dry weight sediment. The results indicate that CuO NPs have higher effects on growth, feeding rate, and reproduction of *P. antipodarum* than micro CuO.

Buffet et al. (2011 and 2012) studied two marine invertebrates (*Scrobicularia plana* and *Hediste diversicolor)* after exposure to CuO NPs (10µgL^{-1}) and soluble salt form of Cu ($CuNO_3$) over a 21 days period, in order to evaluate behavioural and biochemical responses. They found a significant increase in superoxide dismutase activity (SOD) in animals exposed to CuO NPs. Levels were significantly higher compared to controls and animals exposed to soluble Cu. Catalase activity (CAT) increased significantly in both species upon exposure to CuO NPs (as compared to controls), whereas only catalase (CAT) activity was observed to have increased significantly in *H. diversicolor*. Exposure to soluble Cu brought no significant changes to lipid peroxidation levels.

Griffitt et al. (2007 and 2009) examined the acute toxicity of nano (80nm) and soluble copper ($CuSO_4$) on the gill of zebrafish (*Danio rerio*) by observing the behaviour of Cu NPs in terms of bioavailability, aggregation and dissolution of these particles in aqueous media. The results showed that Cu NPs are acutely toxic to zebrafish (with a 48 hours LC_{50} concentration of 1.5mgL^{-1}). The toxicity occurred primarily at the gills, and Cu NPs showed gradual aggregation suspension in water (50–60% of total added mass was removed from the water), the observed toxicity cannot be explained by the aggregation

and dissolution of the particles alone, but the size of these particles may play a key role in the CuO NPs toxicity.

1.3.3 Toxicity of CuO NPs in mussel organisms

Gomes et al. (2011 and 2012) assessed the genotoxic effects of CuO NPs and ionic form of Cu on two types of cells (gills and digestive gland) in mussels (*Mytilus galloprovincialis*). They measured oxidative stress (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), lipid peroxidation (TBARS), metal exposure surrogate (metallothioneins (MT)). *M. galloprovincialis* mussels were exposed to 10μgL^{-1} of CuO NPs and the ionic form of Cu for 15 days. The results indicated that CuO NPs caused oxidative stress indicated by rising SOD, CAT and GP_X activities in the cells of *Mytilus galloprovincialis*, while ionic Cu caused no significant changes or increases to enzymatic activities in *M. galloprovincialis*. However, CuO NPs and ionic Cu both contributed to lipid peroxidation in exposed mussels.

Gomes et al. (2013) followed up their previous work with a study comparing CuO NPs and Ag NPs with their respective bulk forms (Cu and Ag MPs). They tested toxicity and DNA damage to the haemolymph cells of *Mytilus galloprovincialis* mussels, resulting from copper oxide NPs and copper ions (Cu). The outcomes of this study demonstrated both forms of Cu and Ag metals (NPs and ionic) cause DNA damage to haemolymph cells, and that exposure time influenced the toxicity of the metal NPs. However, larger particles of Cu and Ag showed higher genotoxicity than NPs, suggesting different mechanisms of action that may be mediated through oxidative stress, and DNA damage was presented as an important biomarker reflecting the effects of genotoxicity in CuO and Ag NPs in marine molluscs.

Recently, Gomes et al. (2014) expanded his study on mussels and CuO NPs toxicity by investigating changes in protein expression profiles in mussels *Mytilus galloprovincialis* exposed for 15 days to CuO NPs and ionic form of Cu^{2+} (10μgL^{-1}) using a proteomic approach (Two-dimensional gel electrophoresis and mass spectrophotometry procedures). This study reported that CuO NPs and Cu^{2+} induced major changes in protein expression in *Mytilus galloprovincialis* indicating several tissue and metal-

dependent responses. However, CuO NPs showed a higher tendency to up-regulate proteins in the gills and down-regulate in the digestive gland, while Cu^{2+} showed the opposite tendency. The conclusion of this study indicated that the toxicity of CuO NPs is not only due to Cu ions dissolution and can result in mitochondrial and nucleus stress-induced cell signalling cascades that can lead to apoptosis.

Hu et al. (2014) assessed CuO NPs toxicity (100nm) (400, 700 and 1000ppb CuO NPs) in blue mussels *M. edulis* by measuring protein oxidation for different tissues (digestive gland, gill and mantle) using six unique proteins (alpha- and beta-tubulin, actin, tropomyosin, triosephosphate isomerase and Cu–Zn superoxide dismutase) using the Bradford method, assessing the lysosomal membrane stability in haemolymph using the neutral red retention time (NRRT) assay. Results showed that, two spots (actin and triosephosphate isomerase) showed decreased protein thiols, while three (alpha-tubulin, tropomyosin and Cu‑Zn superoxide dismutase) showed increased carbonylation which indicating protein oxidation of cytoskeleton and enzymes in response to CuO NPs exposure. Furthermore, CuO NPs affect lysosomal membrane stability, confirming the usefulness of the NRRT assay as a bioassay for NP toxicity in aquatic species such as *M. edulis.* This study concluded that CuO NPs encountered in the natural environment have the potential to affect filter-feeders across multiple levels of biological organisation.

In addition, Hanna et al. (2014) examined the CuO NPs toxicity in marine mussels (*Mytilus galloprovincialis)* and the influence of mussels on the fate and transport of CuO NPs. In this study, *M. galloprovincialis* exposed to 1, 2, or $3mgL^{-1}$ CuO NPs for four weeks, and then examined the clearance rate, rejection, excretion and accumulation of Cu, and mussel shell growth. Results indicated that CuO NPs decreased the clearance and growth rates in exposed mussels at $3mgL^{-1}$ CuO NPs (clearance rate was 48% less and growth was 68% less). *M. galloprovincialis* rejected and excreted CuO NPs in bio deposits containing as much as 110mg Cug^{-1}, suggesting the potential for magnification in sediments. This study demonstrated that filter feeder such as mussels can impact the fate and transport of CuO NPs in aquatic environment and potentially cause magnification of CuO NPs in mussel bed communities, creating a significant source of Cu to marine benthos.

1.4 Experimental organisms

The marine environment is considered the ultimate sink for environmental contaminants, due to its receipt from various sources of these chemicals. This directly damages aquatic habitats, and affects the animals that depend on these habitats (Valavanidis et al., 2006). Nanoparticles can enter the aquatic environment from various sources and routes, natural sources, such as volcanic eruption and soil degradation, as well as anthropogenic sources, including wastewater discharge and oil production (Gardner, 1995, Handy et al., 2008 and Blinova et al., 2010). Many species have been used specifically to investigate different sources of pollution including NMs. These studies have variously investigated bluegill sunfish (*Lepomis macrochirus*) (Choi and Oris 2000), Atlantic flounders (*Platichthys flesus*) (Fessard and Livingstone 1998), juvenile carp (*Cyprinus carpio*) (Zhao et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Arndt and Wagner 1997) and the blue mussel (*Mytilus edulis*) (Koehler et al., 2008).

Large number of the studies concerned with the monitoring and assessment of chemical contamination (both field and laboratory studies) in the marine environment have agreed that invertebrate organisms (such as crustaceans, and bivalve molluscs) offer a unique target group for assessment of potential contamination; this includes studies regarding the assessment of toxicity from NMs. This is because invertebrates represent around 95% of aquatic species and are important within marine systems, feeding directly on bacteria, algae or organic matter, and providing a trophic link to higher levels in the food chain. These organisms, from the individual animal to the entire invertebrate community, perform important ecological function, and some exhibit high sensitivity to contaminants in the form of, for example, oxidative stress (Hodkinson and Jackson 2005; Baun et al., 2008; Canesi et al., 2012).

1.4.1 Mussels as bioindicator organisms

Bivalves such as mussels are one of the best common bioindicator organisms for detecting stress, levels of pollution and toxicity in the aquatic environment. They are particularly suited to the study of the toxicity of particulate materials because of the way they feed, by actively filtering the water that contains microalgae, bacteria, contaminants and particles through their gills, resulting in the accumulation of high concentrations of contaminants and particles in their tissues (Valavanidis et al., 2006; Yarsan et al., 2007; Julshamn et al., 2008).

In addition, another advantage of using these species as a bioindicator is that mussels are widely distributed worldwide, and can live in the same area for decades; therefore, they can provide reliable data on the long and short-term effects of contamination. They can also adapt to variable environmental conditions (for example temperature and salinity), and external threats (such as contaminants and particles), and are easy to sample. Moreover, some species are an important food source for humans, especially in coastal areas (Figure 1.4) (Dailianis et al., 2003; Angelo et al., 2007; Kucuksezgin et al., 2008; Tsangaris et al., 2010).

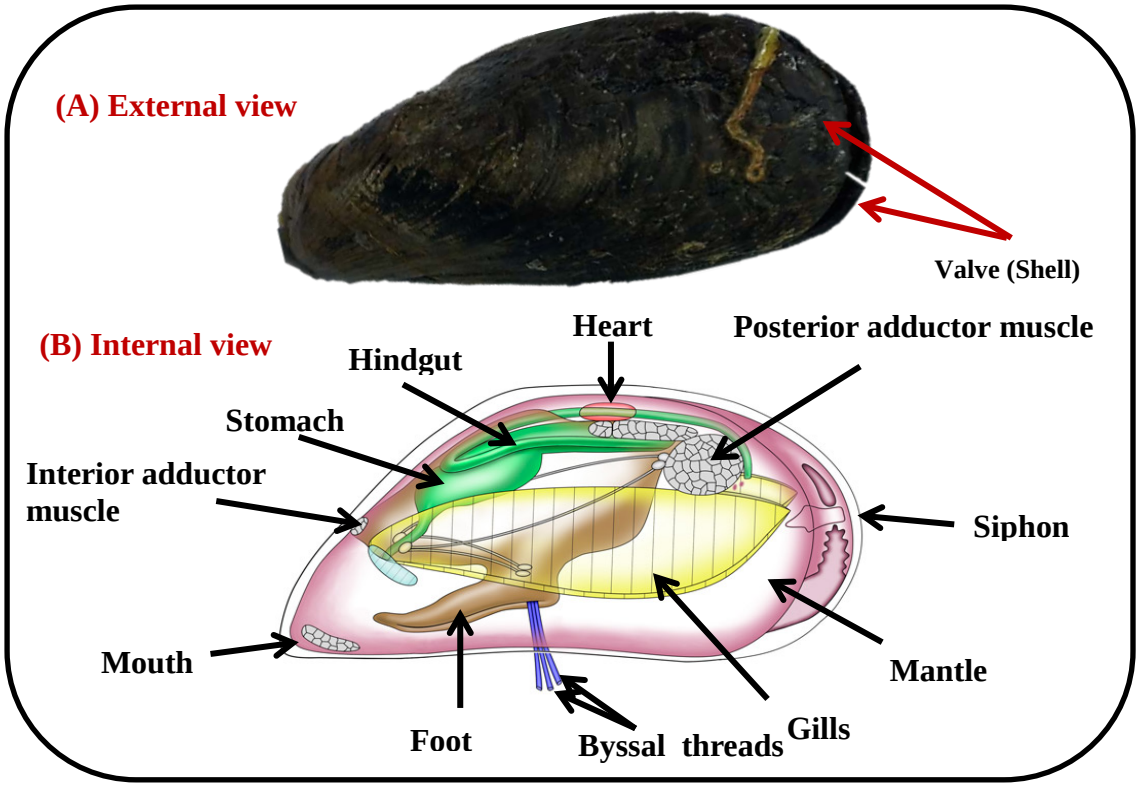


Figure 1.4: The morphology of mussel species (*M. edulis*).

Mussels have two valves, which are produced in almost equal shape and size by the underlying mantle, which also has reproductive and energy storage functions. The foot is an essential organ enabling the mussel to attach itself by byssus threads to a solid substrate; sometimes these threads break and are replaced, enabling mussels to relocate passively within clumps or in rock cracks (Bayne, 1976; Beaumont et al., 2006; Jouvet 2010). Both feeding and respiration take place via the mussel gills. Mussels filter the water column to capture their food (i.e. organic particles, such as bacteria, phytoplankton and other organic matter), which is then carried towards the stomach. However, mussels have an effective sorting mechanism and some particles are rejected as pseudo-faeces before they enter the gut (Beaumont et al., 2008; Alshaeri et al., 2013). In addition, the particular reasons for selecting these two specific of mussels (*Mytilus edulis and Modiolus modiolus*) as bioindicators for this study are as described below.

- The difference in habitats and related environmental conditions, such as temperature various and the levels of sunlight, which may contribute to the threat to the mussel species. *M. edulis* lives in a community in intertidal areas in shallow water, whereas *M. modiolus* lives either in communities or individually in subtidal areas in water around 20 m deep.

- The difference in mussels size (shell length), which may influence the mussels' tolerance to changes in environmental conditions (temperature and salinity) and external stress (contaminants and NMs) (especially *M. modiolus). M. edulis* has an average size of between 5 and 10 cm, whereas *M. modiolus* is up to double this size, ranging between 15 and 20 cm.

- In particular, *M. modiolus* beds are recognised as priority marine features in Scotland's coastal marine environment, providing habitats that support an enormous biodiversity, and as such are an example of as biogenic reefs under the Habitats Directive description of reefs (Mair et al., 2000). In addition the longevity of *M. modiolus* and their ecology could make them an important indicator species for potential impacts of NMs in the marine environment. Despite the importance of *M. modiolus* for making habitats and as valuable indicators of past environmental stress, *M. modiolus* has been out of the attention from most of ecotoxicology studies

compared to other mussel species such as *Mytilus edulis* (Anwar et al., 1990). Hence, increasing the attention on this essential species, in particular the study of potential NM toxicity is significantly vital in order to understand possible threats that may occur, which could influence negatively *M. modiolus* bed habitats and therefore the vast biodiversity living within these habitats.

1.4.2 The blue mussel (*Mytilus edulis*)

The blue mussel, also called the common mussel, encompasses three recognised species that are very similar to one another, with a few genetic and morphological differences, *Mytilus trossulus, Mytilus galloprovincialis and Mytilus edulis* (Bayne 1976; Toro et al., 2004; Beaumont et al, 2008). *M. edulis* is a mussel species that lives in intertidal areas, and attaches to a diversity of substrata, such as rocks, fixed sediments, artificial substrates, dead shells, shingle, and even compacted sand or mud (Figure 1.4 and Figure 2.1a) (Seed and Richardson 1990). *M. edulis* is an active filter feeder, pumping water across its gills. Under ideal conditions, it can processes on average 7 litres of seawater per hour (Jouvet 2010), which means this species is subject to potential threats from chemicals dissolved in the water or particulate matter, including NMs. Of note is the fact that NMs can be transferred through the microorganism living in the water column, such as algae as a primary source of food for mussels (Beaumont et al, 2008; Baun et al., 2008).

M. edulis are the most abundant mussel species in Europe, North Pacific and North Atlantic oceans; they live along the west coast of Europe from France in the south to the Shetlands in the north (Beaumont et al. 2006 and 2008). Within the UK, *M. edulis* is most extensively present around the west coast of Scotland, north Wales, and the estuaries of south-west England. *M. edulis* also occurs in other parts of the world, such as the White Sea, the North African coast, the Mediterranean, the coasts of Chile, Argentina, the Falkland Islands and the Kerguelen Isles (Gardner, 1995; Innes and Bates 1999; Tora et al., 2004; Beaumont et al., 2008).

1.4.3 The horse mussel (*Modiolus modiolus*)

The horse mussel, *Modiolus modiolus* (Figure 2.1b) is the second mussel species used in the current study and live individually in benthic habitats, as well as forming aggregations by attaching to rocks with byssal threads; they have an important stabilising effect on the seabed (Rees et al., 2009). *M. modiolus* individuals are among the largest European mussels and can grow up to 200 mm. The long-lived feature of the species (over 45 years) enables researchers to study it to attain reliable evidence of patterns of past environmental change affecting marine ecosystems (Anwar et al., 1990; Rees et al., 2009; Roberts et al., 2011).

M. modiolus is widely distributed in the Northern Hemisphere, existing on the Atlantic coast of North America, from Labrador to South Carolina and in the Pacific (Bering Sea, Japan and west coast of North America) (OSPAR Commission 2008). Within the UK, it is most commonly present in northern and western areas. The species has been recognised as an essential element in Scotland's near shore marine environment (Mair et al., 2000), because *Modiolus* beds provide a suitable habitat for a wide range of flora and fauna; their assemblages are also known as biogenic reefs according to the Species and Habitats Directive description of reefs (Mair et al., 2000). Within Europe, the species lives in the Barents Sea, Iceland, Norway and the White Sea to the Bay of Biscay and is present in the Skagerrak and Kattegat, the Wadden Sea and along the coast of France (OSPAR Commission 2008).

1.5 Biomarkers and approaches

1.5.1 Cell viability

Cell viability is one of the essential biomarkers used in cytotoxicity studies. It is determined by assessing live and dead cells by applying specific dyes (Davey and Kell 1996). Many methods have been developed for assessing and counting biological cells in the last few decades, such as a trypan blue and flow cytometry (FC). The trypan blue technique is a traditional dye exclusion test that uses trypan blue dye to identify dead cells under the microscope (Absolom 1986). This technique was used in this study due to its simplicity, low cost, quick results and small number of cells required. FC is a well-known method for analysing the expression of cell surface and intracellular molecules, characterising and defining different cell types, allowing a simultaneous multi-parameter analysis of single cells (Diaz et al., 2010).

FC is a cell analysis technique used for counting and analysing a wide range of biological cell types as particles in heterogeneous cell populations. It involves assessing the purity of isolated subpopulations, and analysing cell size and volume using multi-parametric analysis of various cell parameters, based on light-scattering and fluorescent signals (Ormaerod 1994; Diaz et al., 2010). The FC method has been widely applied with biotechnological applications, and has influenced biotechnology significantly due to its valuable features. For example, the ability of this approach to count a high number of cells and particles (in widely available cytometers the count rate is 50,000 cells per second, whereas it is up to 100,000 cells per second in specialised instruments) in one run, high analysis rate, quick results, sensitivity, is less time consuming and more precise (Ormerod 1994; Di Carlo et al., 2010; Diaz et al., 2010).

Several FC techniques have been developed to suit different study purposes, ranging from cell counting and identification to bioprocess control and prediction in the development of more accurate kinetic models, such as forward scatter cytometry (FSC) and side scatter cytometry (SSC) (Diaz et al., 2010; Zucker et al., 2010). The forward scatter cytometry (FSC) mainly measures the overall size of the cells, and cellular size comparisons; however, some small particles can absorb FSC if they have an irregular

cell shape, cell nucleus, other organelles and damage to the cell membrane. The SSC is considered useful for providing information on internal structures and organelles.

The common flow cytometer has several basic operational units: a light source, the flow cell and the hydraulic fluidic system, several optical filters to select specific wavelengths, and a group of photodiodes or photomultiplier tubes to detect signals of interest, and finally a data processing unit (Diaz et al., 2010) (Figure 1.5). When employing this technique, samples, such as blood cells, are passed through a narrow stream, which allows alignment with optical excitation to serially examine those cells of interest according to fluorescence or scatter signatures (Ormaerod 1994; Di Carlo et al., 2010).

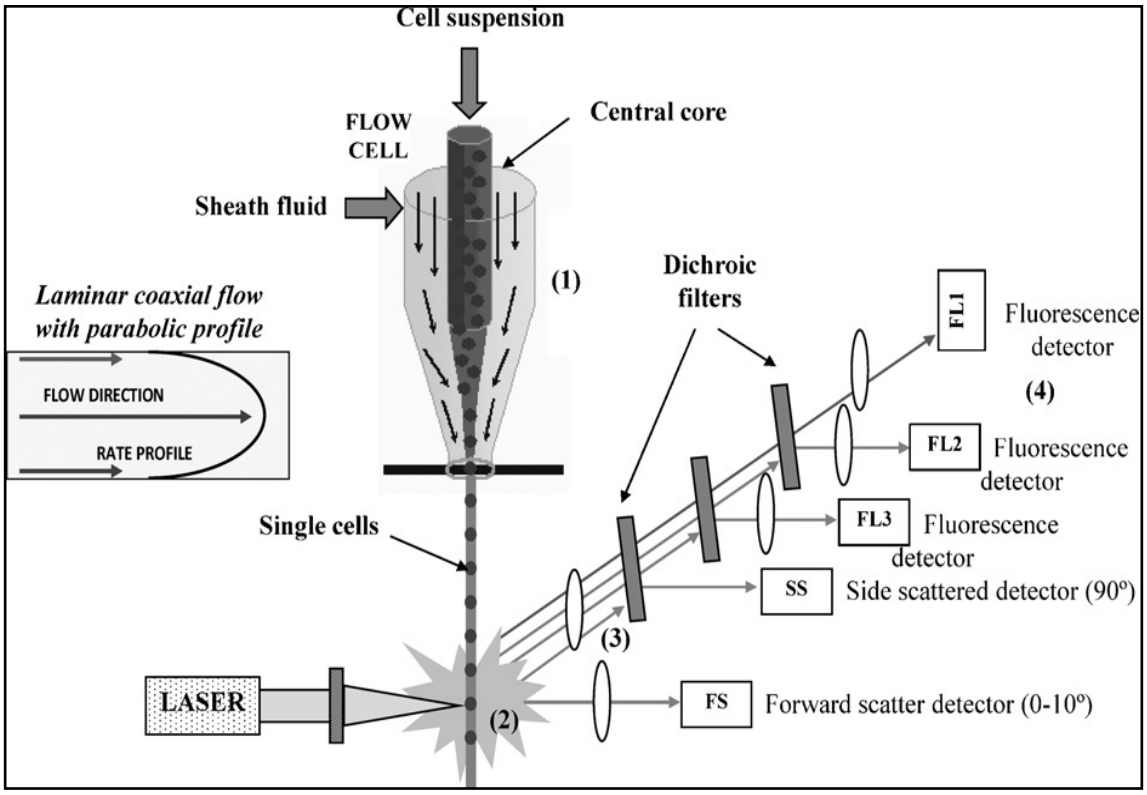


Figure 1.5: Scheme of a typical flow cytometer (Diaz et al. 2010).

1.5.2 Oxidative stress

The term "oxidative stress" refers to an imbalance in an organism's antioxidant defences that affect the neutralisation of oxygen free radicals and nonradical reactive species (referred to as reactive oxygen species; ROS). This disturbance can seriously damage the biological functioning of cells, potentially resulting in damage to DNA, proteins and lipids (Halliwell 1993; Valavanidis et al., 2006). Living organisms regularly generate oxidants as a normal by product of aerobic metabolism but can be produced at elevated rates under pathophysiological conditions (Figure 1.6) (Sies 1997; Gaté et al., 1999; Livingstone 2003).

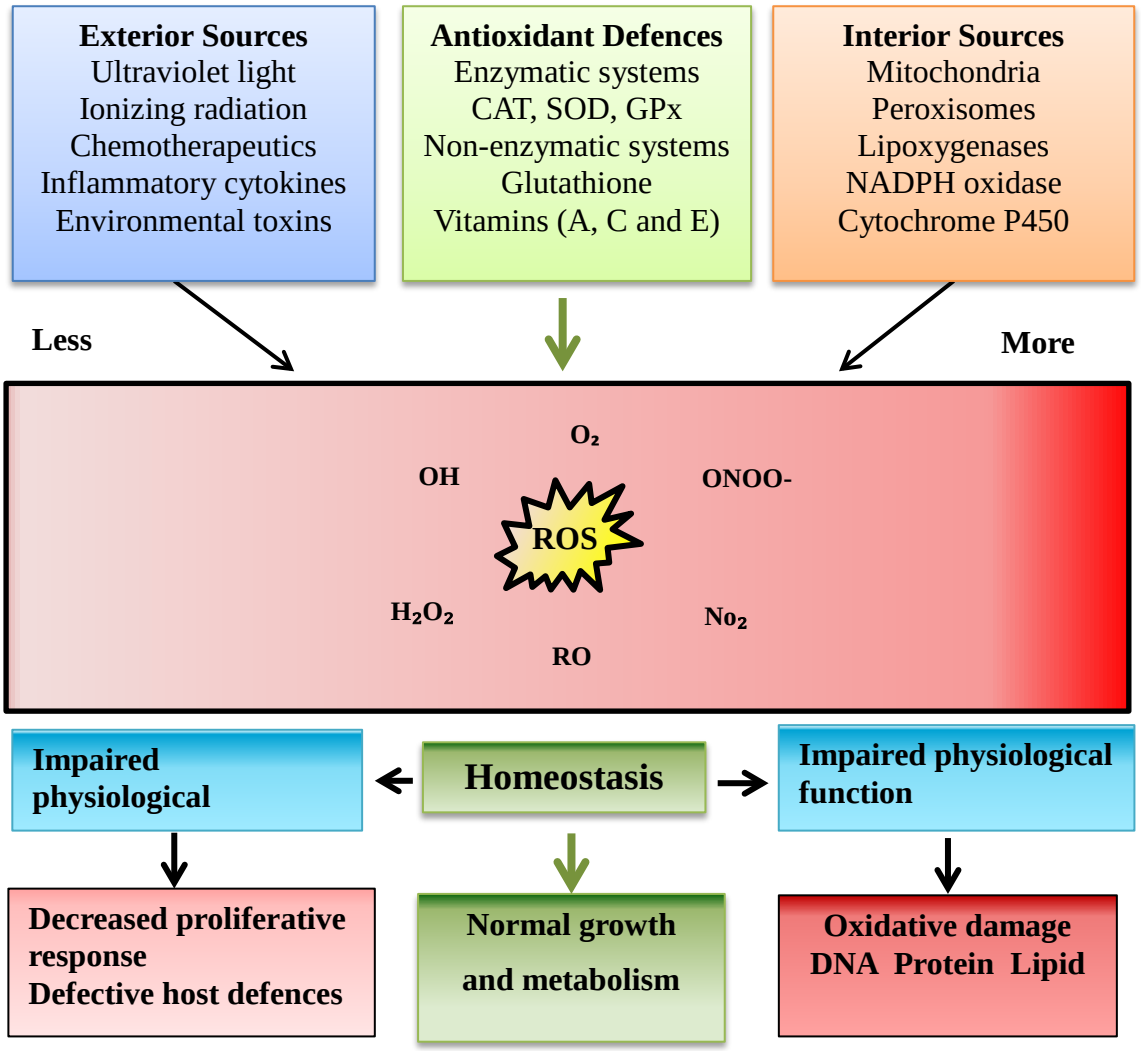


Figure 1.6: The oxidative stress mechanism. (Image adapted from Finkel and Holbrook 2000).

In oxidative stress, some of the unstable free oxygen molecules fail to reduce to water (H_2O) during the metabolic processes, which then results in the formation of reactive oxygen species. These include superoxide anion radicals (O_2^-), nitric oxide (NO^-), peroxyl radical (ROO^-), hydroxyl radical (HO^-), and non-radical species such as the proxidant species hydrogen peroxide (H_2O_2), which cause oxidative damage to the cellular components of the organism (Storey 1996; Gaté et al., 1999; Jackson and Loeb 2001). Each ROS has unique properties and reactivity; for example, hydrogen peroxide is freely diffusible and relatively long-lived, while others, such as superoxide anion radicals and hydroxyl radicals are highly unstable and reactive (Finkel and Holbrook 2000). During the evolution of biological systems in organisms, many antioxidant defence enzymes also evolved. Their role is to maintain a state of balance between the generation of ROS and antioxidant defences, which reduces the level of oxidative stress. These enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP_X) (Ames et al., 1993; Valavanidis et al., 2006).

There are three forms of superoxide dismutase (SOD), including copper zinc superoxide dismutase (Cu/ZnSOD), also known as (SOD1), manganese superoxide dismutase (MnSOD), also known as (SOD2), and extracellular superoxide dismutase (EC-SOD), also known as (SOD3). The key function of SOD is to reduce the oxidative damage by converting the superoxide anion (O_2^-) to a less reactive H_2O_2 (Gaté et al., 1999; Fattman et al., 2003). Catalase (CAT) is another antioxidant enzyme mainly acts in cellular detoxification, which converts the H_2O_2 produced by SOD to water (H_2O) and O_2, and finally there is a third enzyme, called glutathione peroxidase (GP_X), that acts in an insoluble form transforming H_2O_2 to water via the oxidation of reduced glutathione (GSH) in oxidized glutathione (GSSG) (Gaté et al., 1999).

The impact of oxidative stress on living organisms has been studied and reviewed extensively over recent decades. Studies have examined lipid peroxidation, and damage to DNA and proteins (Halliwell 1993; Ames et al., 1993; Storey 1998; Gate et al., 1999; Betteridge 2000; Dröge 2002). This type of damage is considered a key factor in numerous diseases; for example, diabetes, atherosclerosis, the aging process, cancer, apoptotic cell death (particularly in the nervous system), blood pressure,

neurotransmission, the generation of insulin resistance, cardiovascular disease, and other chronic diseases (Regoli 1998; Ceconi et al., 2003).

During the last 30 years, techniques for measuring oxidative stress have been developed for medical research and modified for environmental use, to deliver methods that capable for producing reliable and accurate results, reflecting the causes of lipid peroxidation and damage to proteins and DNA in biological systems. For example, lipid peroxidation can be measured by assessing malondialdehyde (MDA) as a by-product of lipid peroxidation using thiobarbituric acid reactive substances assay (TBARS). Researchers use this method widely, due to its capability to detect small changes in MDA and to produce reliable results (Valavanidisa et al, 2006). In addition, many assays have been used to assess levels of oxidative stress, by measuring the activity of antioxidant defence enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxide (GP_X) (Ames et al., 1993; Ceconi et al., 2003).

1.5.3 Comet assay

Studies utilising new approaches to detect DNA damage in a cell have increased in popularity in the last few decades (Rojas et al., 1999). Many techniques have been found viable for assessing DNA damage, such as the micronucleus test (MNT), the sister-chromatid exchange assay (SCE) and the chromosomal aberration assay (CA) (Kim and Hyun 2006). However, not all these assays are suitable for use with a wide range of cells, as these have been engineered purely for use with mammalian cells, for instance, metaphase techniques that includes sister chromatid exchange (SCE) and chromosomal aberration assays were not suitable for many fish species (Salmonids and Cyprinids) because of the fish karyotype consists of large numbers of small, irregular chromosomes (Al-Sabti and Metcalfe 1995).

An effective and comprehensive method for assessing DNA damage is known as Comet assay. The word “comet” describes the individual cell DNA migration patterns produced by this method (Tice et al., 2000). Östling and Johanson (1984) first introduced the technique in 1984, to detect double-stranded DNA breaks at the level of individual cells (Fairbairn et al., 1995; Rojas et al., 1999; Tice et al., 2000). The cells, once embedded in agarose, are placed on a microscope slide. The cells are lysed by

detergents and salt treatment and the liberated DNA electrophoresed under neutral conditions (pH 7), ensuring the detection of double-stranded DNA breaks only (Rojas et al., 1999; Tice et al., 2000).

Singh et al. (1988) has further developed this method by using Alkali-labile sites (ALS) (pH >13) to analyse the detection of single-stranded DNA breaks, and named it single cell gel electrophoresis (SCGE) (Singh et al., 1988; Fairbairn et al., 1995; Collins et al., 1997; Rojas et al., 1999; Tice et al., 2000). More recently there have been several modifications and developments to both versions of Comet assay (neutral and alkaline). In the last few years, adaptations have rendered it useful for testing cells of a diversity of species, including bivalves and other invertebrates (Woods et al., 1990; Klaude et al., 1996; Coughlan et al., 2002; Rank and Jensen 2003; Hartl et al., 2004; Hartl et al., 2010).

The SCGE, or Comet assay approach, has been widely accepted in many laboratories and field studies, due to its applicability to a wide range of different cells (for example, fish, mussels and human cells) (Singh and Hartl 2012). In addition this technique has many advantages: i.e. the availability of equipment, ease of use, simplicity, low cost, flexibility, the possibility of conducting studies using relatively small amounts of a test substance, sensitivity when assessing low levels of DNA damage, the requirement for only a small number of cells (50-1000 per sample), reliable results and short time required to complete the experiment (Woods et al., 1999; Collins et al., 1997; Tice et al., 2000; Hartmann et al., 2003; Akcha et al., 2004; Klobucar et al., 2008; Dhawan et al., 2009; Singh and Hartl 2012).

The comet assay involves several steps. The technique first embeds a suspension of nucleated single cells (may require a step for extracting cells from the complex tissues of organs) into an agarose sandwich (NGA and LMP) on a microscope slide, removing the cell membranes by placing the slides into a lysis solution, under electrophoresis conditions to unwind the DNA. This is followed by the neutralisation and staining of the cells with Gelred and measuring DNA damage under a Zeiss Axiophot microscope using live video scoring with Comet Assay IV (from Perceptive Instruments) (Figure 1.7) (Woods et al., 1999; Rojas et al., 1999; Tice et al., 2000; Hartmann et al., 2003; Singh and Hartl 2012).

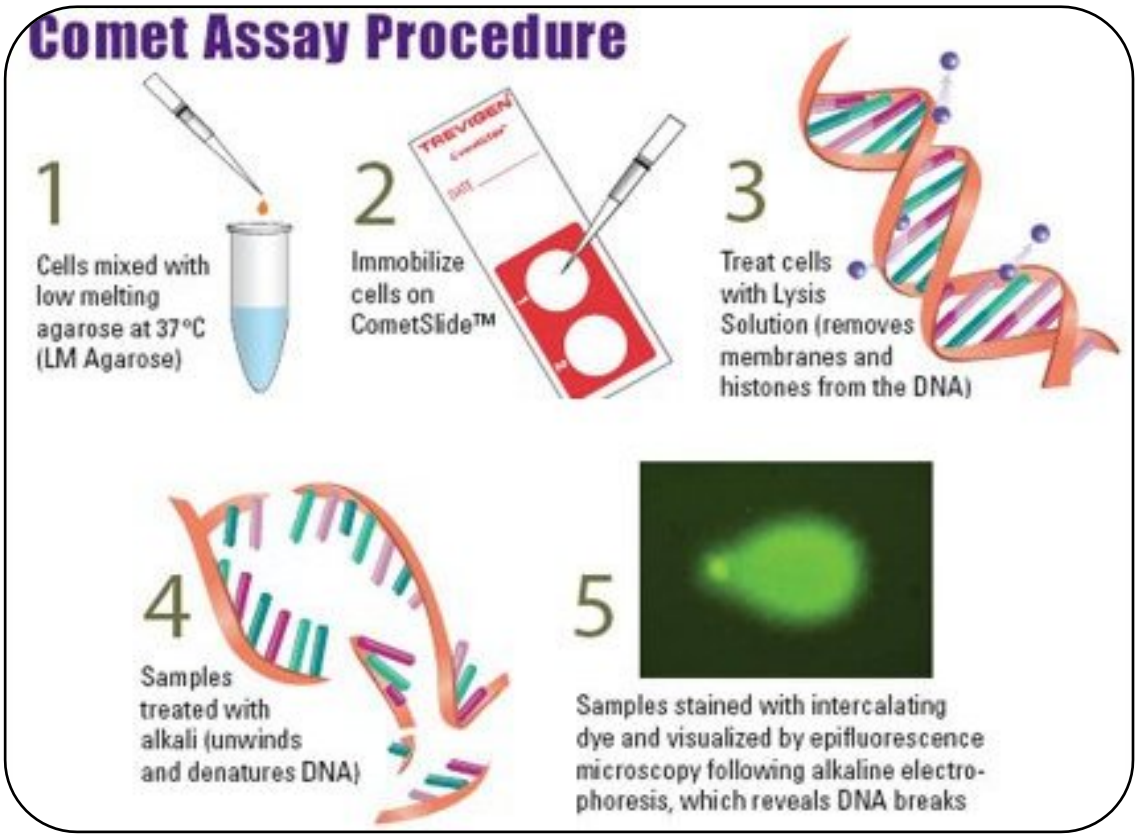


Figure 1.7: Comet assay procedure summary (Amsbio 2010).

1.5.4 Superoxide dismutase (SOD)

Reactive oxygen species (ROS) are continually generated as by-products during the cell respiration mechanism in organisms, which may lead to various impacts on the cells, including an oxidation of DNA bases, lipid peroxidation, protein degradation, and the inactivation of enzymes (Halliwell 1993; Zelko et al., 2002; Grayck et al., 2005; Almeida 2005). A variety of antioxidant defence enzymes are produced by cells to protect against ROS generation, these include metal sequestering proteins, use of compounds such as vitamin C, vitamin E, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Fattman et al., 2003).

Superoxide dismutase (SOD) is an antioxidant defence enzyme that provides essential protection against uncontrolled reactions with oxygen based radicals to biological cells by catalysing the dismutation of two superoxide (O_2) radicals into hydrogen peroxide (H_2O_2) and oxygen (O_2) (Keller et al., 1991; Carpo et al., 1992; Fattman et al., 2003). Thus far, three isoforms of SOD enzymes are identifiable in living cells. These are

commonly classified according to the metals they contain, their type of amino acid sequences, localisation and function. They include copper zinc superoxide dismutase (Cu/ZnSOD) also known as SOD1, manganese superoxide dismutase (MnSOD) also called SOD2 and extracellular superoxide dismutase (EC-SOD) also known as SOD3 (Keller et al., 1991; Carpo et al., 1992; Zelko et al., 2002; Fattman et al., 2003).

McCord and Fridovich (1969) were the first to discover a SOD type enzyme, copper zinc superoxide dismutase (Cu/ZnSOD or SOD1). They observed that SOD1 catalyses the dismutation of the superoxide radical into hydrogen peroxide (H_2O_2) and oxygen (O_2) (Carpo et al., 1992; Zelko et al., 2002; Grayck et al., 2005). Cu/ZnSOD is considered a major intracellular SOD; it has a molecular mass of 32,000 Da and contains one Cu and one Zn ion. It is widely distributed in the cytoplasm, peroxisomes, nucleus and mitochondrial intermembrane space in all mammalian cells (such as rat, mouse and human), and in the periplasmic space of bacteria lysosomes (Keller et al., 1991; Fattman et al., 2003; Valentine et al., 2005).

Manganese superoxide dismutase (MnSOD or SOD2) is another enzyme from the SOD family. It is expressed as a tetramer containing a leader peptide in the endoplasmic reticulum, mitochondrial, secretory vesicles, Golgi elements and nuclear envelope of mammalian cells (such as human, rat and mouse) (Carpo et al., 1992; Zelko et al., 2002; Grayck et al., 2005). Marklund et al. (1982) detected a further new SOD enzyme, which they named superoxide dismutase (EC-SOD or SOD3). This SOD enzyme is an extracellular superoxide dismutase with a molecular weight of 135,000 kDa and minor species-specific variations (human, rat, rabbit and mouse) (Fattman et al., 2003; Grayck et al., 2005). EC-SOD also contains two atoms (Cu and Zn) per subunit and is commonly found as a tetramer in most organisms, although it can be sometimes be found as a dimer, and is present in extracellular fluids, including plasma, lymph and synovial fluid (Fattman et al., 2003; Grayck et al., 2005).

Two main reasons for choosing to measure the SOD activity which indicates oxidative stress in exposed mussel gills (among other enzymes such as CAT and GPx). Firstly, SOD deals uncontrolled radicals by converting the superoxide anion (O_2^-) to a less reactive H_2O_2, which means this enzyme dealing directly with these free radicals and

thus SOD activity then reflects the real level of oxidative stress in exposed cells. Secondly, other enzymes functions are significantly dependant on the production of SOD enzyme which is H_2O_2. Catalase (CAT) converts the H_2O_2 produced by SOD to water (H_2O) and O_2, whereas, glutathione peroxidase (GP_X) transforming H_2O_2 produced by SOD to water via the oxidation of reduced glutathione (GSH) in oxidized glutathione (GSSG).

1.5.5 Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation is important resulting from the increasing level of thiobarbituric acid-reactive substances (TBARS) in biological cell membranes. It is linked to the oxidative stress that occurs when there is excessive generation of free radical oxygen species (Regueiro et al., 1999; Beltran et al., 2003; Oakes et al., 2003). For example, malondialdehyde (MDA) is a well characterized TBAR, the oxidation product of polyunsaturated fatty acids (PUFAs) in lipoproteins (Camejo et al., 1998; Beltran et al., 2003). The increased production of TBARS is an important factor in numerous human diseases; it is also reported as a cause of several inflammatory lung disorders, such as adult respiratory distress syndrome, bronchial asthma, pneumonia, chronic obstructive pulmonary disease. It can also be observed in asymptomatic cigarette smokers (Nowak et al., 2001; Beltran et al., 2003).

TBARS or MDA assay has been used extensively since the 1950s for measuring levels of lipid peroxidation in membranes and biological systems, and also for assessing free radical-mediated oxidation of polyunsaturated fatty acids (PUFA) in both animals and plant membrane species (Du and Bramlage 1992; Camejo et al., 1998; Hodges et al., 1999; Butterfield and Laduerback 2002). The TBARS assay has become a common technique for measuring lipid peroxidation, because it offers several advantages, such as reliable results, lack of expense, sensitivity to small TBARS change in tissue, simplicity, requirement for minimal manipulation to assess a large number of samples (Hodges et al., 1999; Nowak et al., 2001; Schisterman et al., 2001; Almroth et al., 2005).

1.6 Characterisation of Cu particles

The characterisation of natural and engineered NMs is the first essential step to understanding physical and chemical properties such as size, surface chemistry, shape, solubility, dissolution, aggregation and charge etc. These characteristics influence the behaviour of particles in different biological and environmental media and are likely to influence bioavailability and toxicity (Peralta-Videa et al., 2011). Multiple techniques have been applied and developed to characterise the physiochemical properties of NMs, especially size, which is a fundamental factor influencing other NPs properties such as Raman spectroscopy, scanning electron microscope (SEM), transmission electron microscope (TEM), dynamic light scattering (DLS), while there are other techniques that apply for measuring the accumulations and dissolution of NMs in exposure media as well as target organisms. These techniques include inductively coupled plasma mass spectrometry (ICPMS), inductively coupled plasma optical emission spectrometry (ICPOES) and atomic absorption spectrometry (AAS) (Oberdörster 2009).

In the research described here, two different viewing techniques were used to characterise particulate forms of Cu (CuO NPs, CuO MPs). Transmission electron microscope (TEM) was used to determine the size and shape of Cu particles in the working medium (seawater), and dynamic light scattering DLS was used to measure the average sizes and zeta potentials of the agglomerates of the Cu particles (See methods for more detail). These particular techniques have been widely accepted in many laboratories and field studies, due to their simplicity, suitable for all particles and can be used to measure the size and shape or size and zeta potential immediately or in a short time frame to obtain results. These techniques are well known and reviewed elsewhere (e.g. Williams and Carter 1996; Sano et al. 2001; Gao et al. 2003; Powers et al. 2006; Karlsson et al. 2009; Stone et al. 2010).

1.7 Aims and objectives

Due to the rapid increase and development in nanotechnology, nanomaterials and nanoparticles are likely to be released into the aquatic environment, which may adversely affect aquatic organisms (Griffitt et al., 2007). Therefore, it is essential to investigate exposure levels; uptake and effects on key organisms, such as benthic species and filter feeders, since data relate to these systems remain sparse (Karlsson et al., 2009). To date, many researchers have studied the effect of CuO NPs on living organisms, including humans and bacteria. However, only a few studies have assessed the toxicity of CuO NPs on aquatic organisms, mostly focussing on crustaceans (*Daphnia magna*, *Thamnocephalus platyurus*, *Daphnia pulex* and *Ceriodaphnia dubia*), and zebrafish (*Danio rerio*) (Buffet et al., 2011 and 2012; Mortimer et al 2008, 2010 and 2011). The majority of these studies focused on effects in the form of oxidative stress, and for this reason, only limited data are available on genotoxicity, in terms of DNA damage and cell viability.

In contrast, limited data are available on the toxicity of CuO NPs to bivalve species, such as mussels. Previous studies only evaluated the toxicity of CuO NPs on mussels (*Mytilus galloprovincialis)* in regard to oxidative stress, lipid peroxidation and metallothioneins only at concentrations 10µgL^{-1} of CuO NPs for 15 days (Gomes et al., 2011, 2012, 2013 and 2014), recent studies only assessed lysosome membrane stability, protein oxidation and accumulation of copper in different mussel species (*M. edulis* and *M. galloprovincialis)* (Hu et al., 2014; Hanna et al., 2014) (See page 14 and 16 for more details). To the best of my knowledge, no study has investigated and compared the toxicity of copper oxide particles (CuO NPs and MPs) on DNA damage, cell viability and oxidative stress in different mussel species living in different habitats (*Mytilus edulis and Modiolus modiolus*).

It is crucial to conduct studies on the toxicity of CuO NPs to different mussel types at various concentrations, since these species are an important food source to people living in coastal areas, and are likely to be exposed to particulate matter as particle feeders, making them vulnerable to these materials. In addition, given their sedentary life style and the fact that they are relatively long lived, examination of them can indicate overall environmental conditions. In particular, *M. modiolus* beds are recognised as priority

marine features in Scotland's coastal marine environment, providing habitats that support an enormous biodiversity, and as such are an example of biogenic reefs under the Species and Habitats Directive description of reefs (Mair et al., 2000). Despite the importance of *M. modiolus* for making habitats and as valuable indicators for past environmental stress, *M. modiolus* has not been the focus of most of ecotoxicology studies, when compared to other mussel species such as *Mytilus edulis* (Anwar et al., 1990). Hence, increased attention on this important species, in particular the study of NM toxicity, is vital in order to understand any possible threats that may occur, which could lead to influence negatively *M. modiolus* bed habitats and therefore the vast biodiversity living within these habitats.

Based on the literature, it was hypothesised that the unique physical and chemical properties of the nano form of Cu (especially the particle size) contributes significantly to the cytotoxicity and genotoxicity of these particles compared to larger particles from the same metal (micro). In addition, it was hypothesised that *M. edulis* mussels might be more resistant and adaptive to external threats such as pollutants, including NMs exposure than *M. modiolus* due to that fact that *M. edulis* mussels live intertidally and thus are exposed frequently to significant changes in natural environmental conditions (such temperature, salinity and water movement), as well as external pollutants regularly introduced to their environment from surrounding areas. This may make this species more tolerant to these changes and threats than *M. modiolus* mussels that live subtidally and thus live in more stable environment. Finally, it was hypothesised that are likely to be more sensitive to chemical exposures than haemocytes in both mussels, and this is because mussels are filter feeders which use their gills to filter food from the water column leading to gill tissue to be exposed frequently to the particles and other chemicals.

Therefore, the focus of this study was entirely directed toward determining and comparing the toxicity of CuO NPs, compared to other forms of copper on two species of mussels (*M. edulis and M. modiolus*) using multiple biomarkers to assess DNA damage, cell viability, oxidative stress, including lipid peroxidation, following these approaches:

- DNA damage determined using single cell gill electrophoresis or comet assay;
- Cell viability assessed using trypan blue and flow cytometry (FC);
- Oxidative stress determined by examining superoxide dismutase (SOD) enzyme activity;
- Lipid peroxidation measured by assessing the level of thiobarbituric acid reactive substances (TBARS).

❖ **The specific objectives of this work were**:

- To study the potential genotoxicity of different Cu forms (nano, micro and salt) on both types of mussels (*M. edulis and M. modiolus*) using the biomarkers mentioned above, and perform a comparison between the different copper forms on both mussel types;
- To determine the size and shape of Cu particulates (nano and micro) in the exposure medium (seawater) using transmission electron microscope (TEM);
- To measure the average sizes, zeta potentials and the agglomerates of CuO NPs and MPs in the exposure medium (seawater) using dynamic light scattering (DLS);
- To evaluate the total amount of Cu in the exposure medium (seawater) as well as the accumulation of copper (from the different Cu chemical forms) in mussels tissues using inductively coupled plasma optical emission spectrometry (ICPOES).

❖ **This study aims to answer the two key questions listed below.**

1. Are nanoparticulate forms of Cu more likely to be harmful to marine mussels than larger particulate and salt forms of the same metal?
2. Is one species of marine mussel more vulnerable than the other to the different forms of Cu being studied?

2 MATERIALS AND METHODS

The section will describe the materials and methods used in this study. First, it will explain the stages required to be carried out prior to starting the experiments, including mussel collection, exposure conditions, nanomaterial chemical and cell preparation. All selected techniques and biomarkers used in the current study will be illustrated including dissection of mussels (haemocytes and gill cells), copper particles characterisation (transmission electron microscope (TEM), dynamic light scattering (DLS), inductively coupled plasma optical emission spectrometry (ICPOES), trypan blue and flow cytometry (FC), single cell gel electrophoresis (SCGE) or Comet assay, superoxide dismutase (SOD) assay, thiobarbituric acid reactive substances (TBARS) assay. Finally, the statistical data analysis is explained.

2.1 Mussel collection

Two different species of mussels were selected for this study. The first experimental organism was the blue mussel (*Mytilus edulis*) (Figure 2.1a) and the second was the horse mussel (*Modiolus modiolus*) (Figure 2.1b). The tolerance of both mussel species to the same levels of contamination was studied, with the aim of understanding the influence of their respective life styles on their chemical sensitivity. Both species were collected and used in laboratory experiments. *M. edulis* was collected from Cramond Island, located in the city of Edinburgh, Scotland, United Kingdom (Figure 2.2) using an empty beaker (Vol. 5L). *M. modiolus* was collected in approximately 20m depth from a site on the North West side of Cava Island, Scapa Flow, Orkney Islands, Scotland, United Kingdom (Figure 2.3).

Following collection, mussels were brought to the aquarium at the School of Life Sciences at Heriot-Watt University. *M. edulis* mussels were cleaned and kept in a 45L plastic tank half filled with aerated and filtered natural seawater (Figure 2.4a), while *M. modiolus* were cleaned and divided between several 300L plastic tanks, half-filled with aerated and filtered natural seawater (Salinity: 32-34 ppt, T: 14°C) (Figure 2.4b). Both species were left to acclimatize for 3 to 4 days without feeding (some mussels were

used in the experiments after 4 days and remaining mussels were regularly fed and water was changed regularly and any dead mussels were removed) (Figure 2.4 a & b).

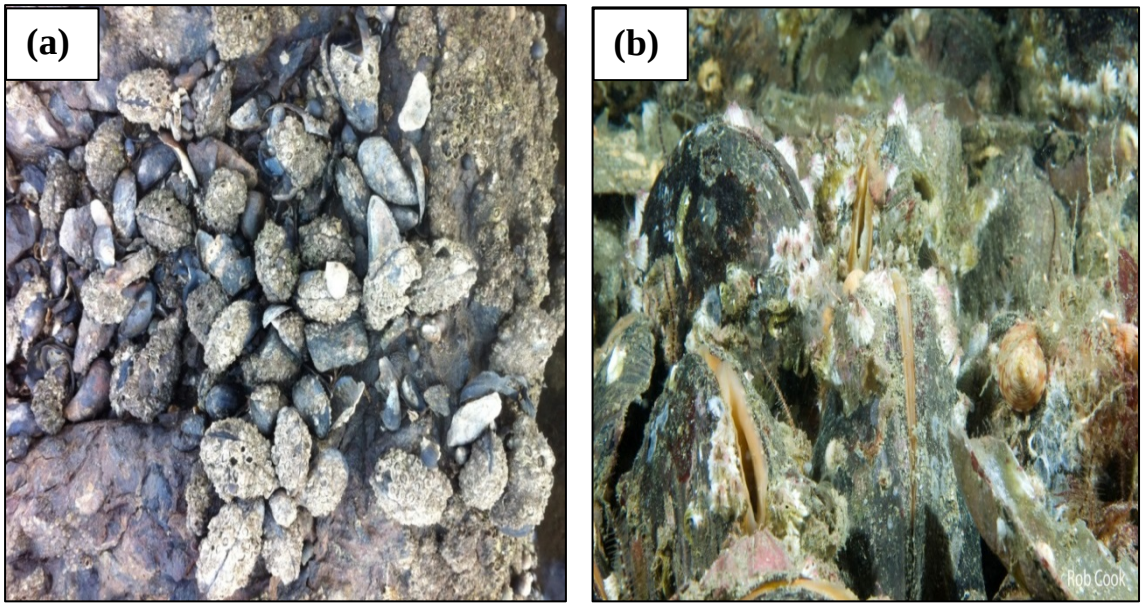


Figure 2.1: Mussels species at their natural habitats. (a) *Mytilus edulis* mussels. (b) *Modiolus modiolus* mussels (Taken by Robert Cook).

Figure 2.2: The collection site for *M. edulis* mussels (Cramond beach).

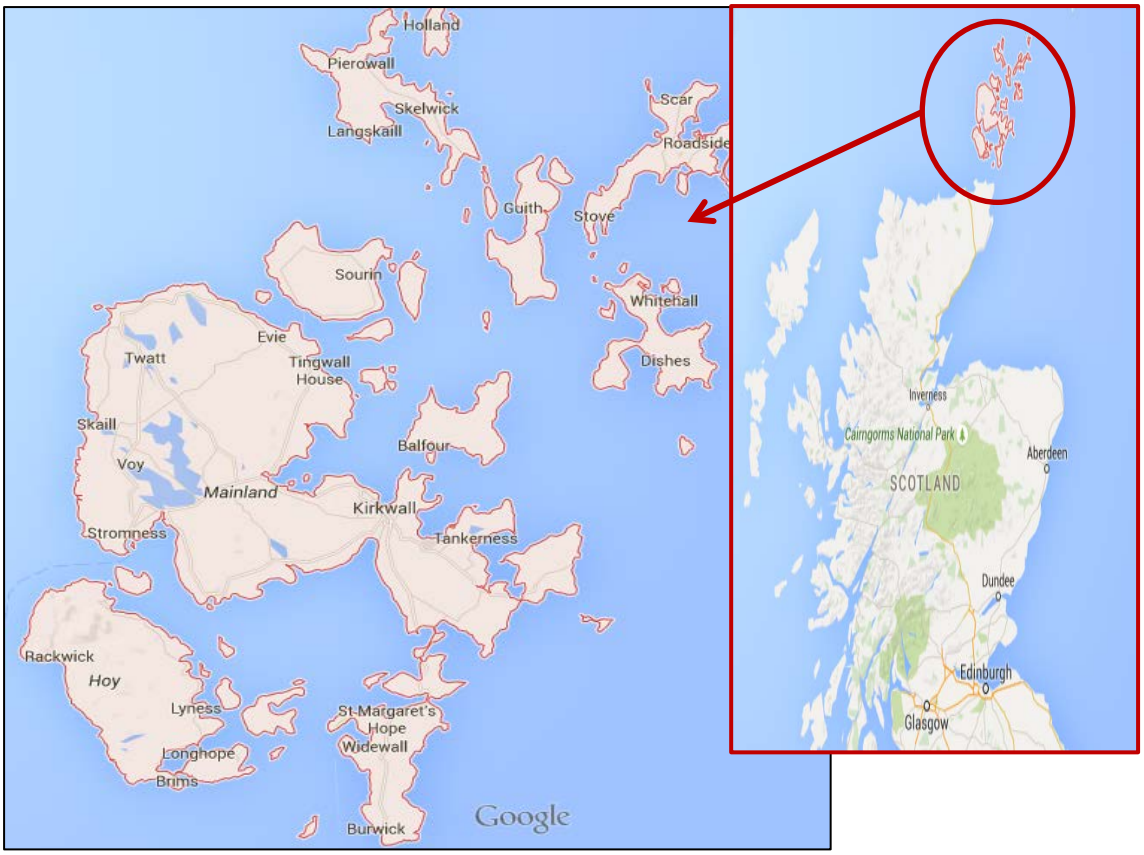


Figure 2.3: The collection site for *M. modiolus* mussels (Orkney Islands).

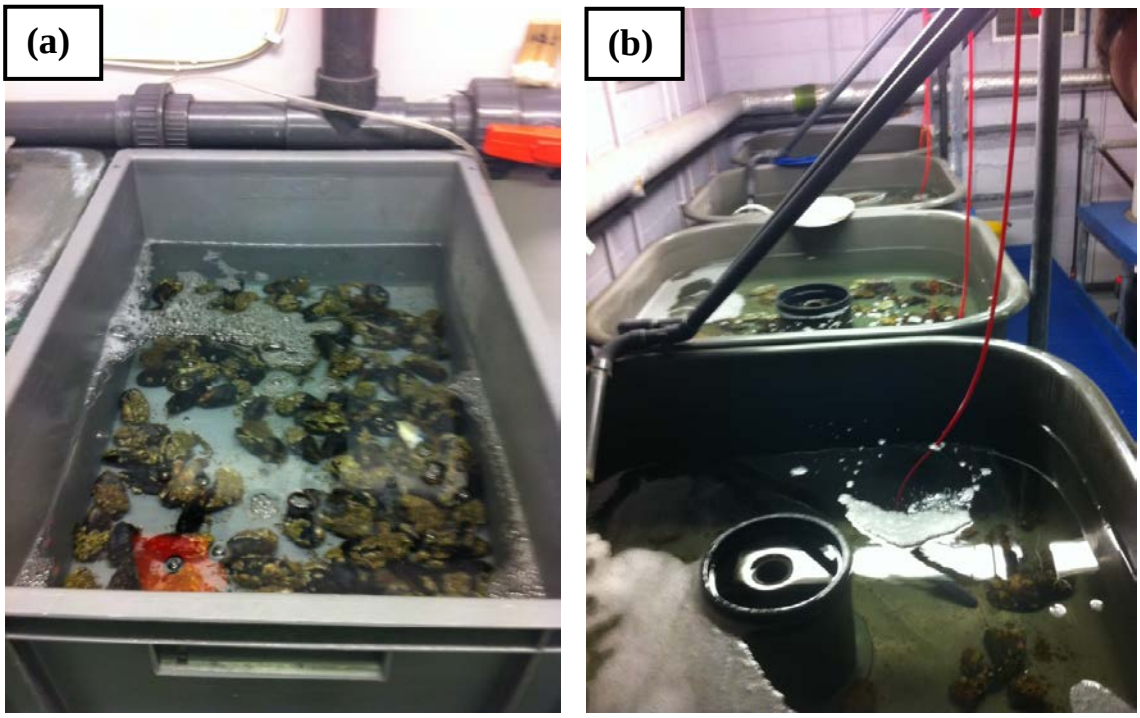


Figure 2.4: Mussels kept in the aquarium. (a) *Mytilus edulis* mussels. (b) *Modiolus modiolus* mussels.

2.2 Exposure conditions

Both mussels were exposed individually to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) using the following nominal concentrations: 5, 10, 15 and 20µgL^{-1}, along with a control that contained aerated seawater only. Five buckets (5L) were prepared (Control, 5, 10, 15 and 20µgL^{-1}), each bucket containing 2L of aerated seawater and 5 mussels from the same species and with the same average size (4.5 – 5.5 cm for *Mytilus edulis* and 9.5 – 10.5 cm for *Modiolus modiolus*). Then all the buckets were spiked with the selected chemicals at the same time and left for 72 hours (exposure period) (Figure 2.5).

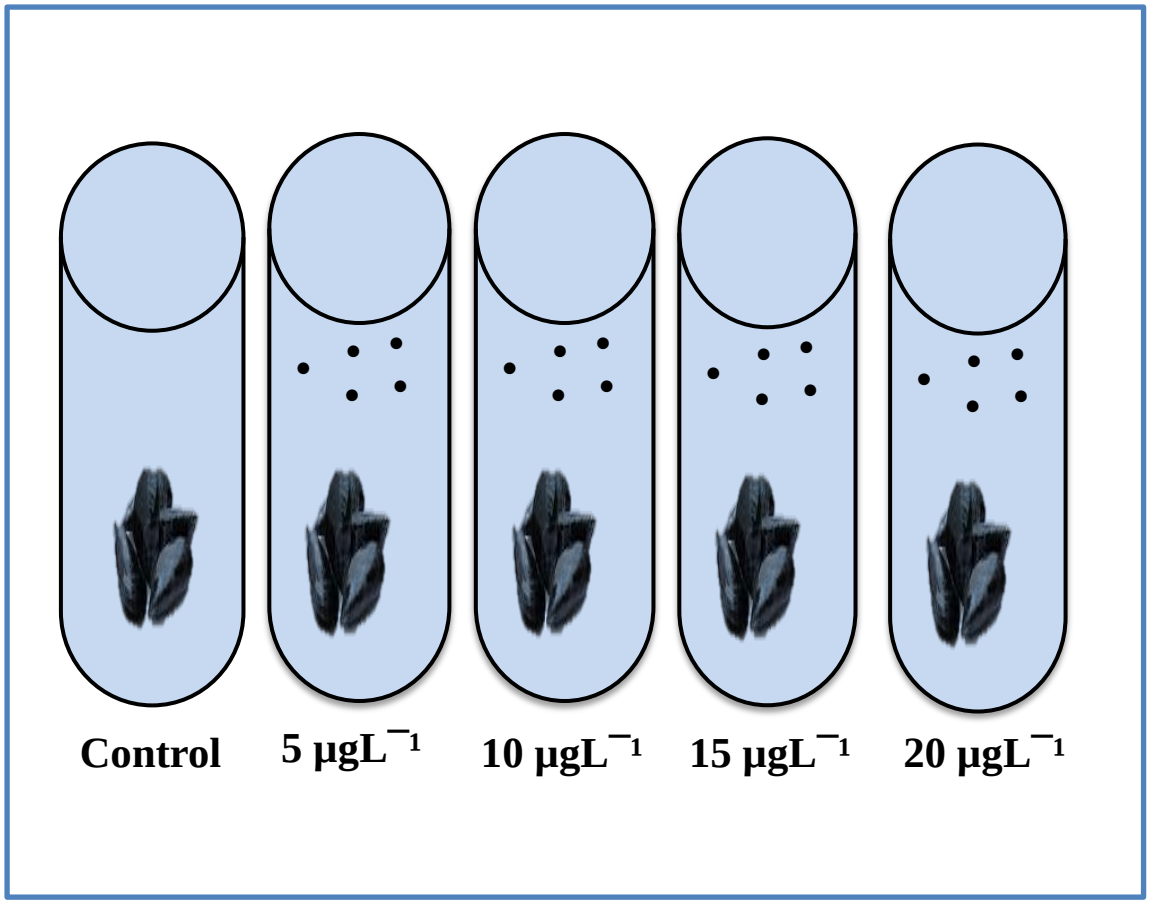


Figure 2.5: Mussels under exposure conditions.

2.3 Chemical preparation

All forms of particulate Cu (NPs and MPs) and salt form ($CuSO_4$) were obtained from a commercial source (Sigma-Aldrich), where CuO NPs & MPs were a black powder and $CuSO_4$ a bright blue powder (Figure 2.6 a, b & c). The particulates' nominal size was <50nm and <5µm, for CuO NPs and CuO MPs, respectively. All Cu chemical stock suspensions were prepared similarly by dispersing 8mg of the selected form of Cu in 100mL of distilled water, and sonicated in a bath sonicator for 30 minutes. Afterwards, 2.5mL of the stock suspension were diluted in 50mL of distilled water (1:20) in order to obtain the concentration of 4 mg/L of the working solution. Then the following equation was used to calculate the final nominal concentrations:

$$V1 = \frac{C2 \ V2}{C1} \qquad (1)$$

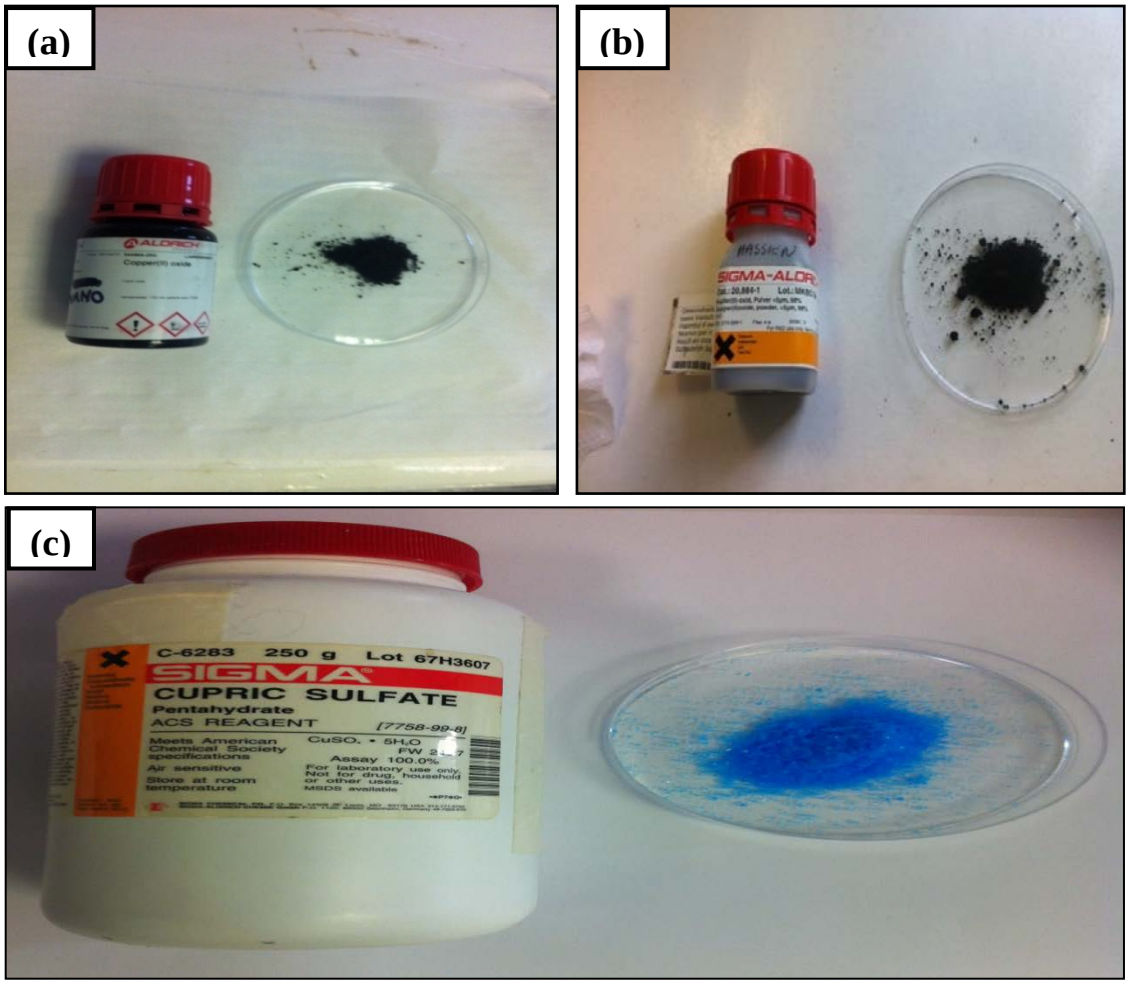


Figure 2.6: (a) Copper oxide nanoparticles (CuO NPs). (b) Copper oxide microparticles (CuO MPs) and (c) Copper sulphate ($CuSO_4$).

2.4 Dissection of mussels

Before starting the selected experiments, mussels exposed to 5, 10, 15 and 20µgL^{-1} of Cu forms and a control group (seawater only) were taken from the exposure buckets (Figure 2.5) and kept in separate beakers to avoid any confusion. All mussels in a given experiment were then dissected to obtain the target cells, haemocytes and gill cells, and this isolation process was carried out on the same day for all mussels in a given experiment (25 mussels for comet, SOD and TBARS assays and 15 mussels for Flow cytometry and Trypan blue experiments) (using the same samples for all experiments so that individual variability is reduced).

2.4.1 Haemocyte preparation

All mussels were placed on their beaks for dissection and scissors inserted between the mussel's valves forcing them open about 5mm apart. This allowed drainage of seawater contained in the mantle cavity (Figure 2.7). Then, osmotically corrected (990mOsmol L^{-1}) Hanks buffered saline solution (HBSS) was prepared by adding 11.1gL^{-1} of sodium chloride (NaCl) to 500mL of HBSS. A 1mL syringe with a 21gauge needle containing 0.1mL of HBSS was inserted into the posterior adductor muscle of each mussel to collect 0.1mL of haemolymph. The needle was removed and the mixture of 0.1mL HBSS and 0.1mL haemolymph cells was gently transferred to an Eppendorf tube and kept on ice or in the fridge for 24 hours and then used for further processing, these cell suspensions remain viable in the fridge for at least 7 days (Hartl et al., 2010).

Figure 2.7: The extraction of haemolymph cells from the mussel.

2.4.2 Preparation of gill cells

The same mussels which were used to extract the haemocytes were also used to extract the gill cells, using the protocol developed by Coughlan et al. (2002) for clams and adapted for mussels by Al-Shaeri et al. (2013). Haemolymph extraction, a scalpel blade was used to cut the adductor muscle and open the valves completely. Then, the gills were extracted from both sides using tweezers and a fresh scalpel blade (Figure 2.8a). The gill from one side was wrapped in a roll of cling film, dipped in liquid nitrogen and stored at -80°C for SOD and TBARS assays. The second gill was prepared for the Comet assay by placing the extracted in a Petri dish containing 2.5mL of HBSS and chopped 20 times using scalpel blades in a scissor-like motion. Then, the mixture of chopped gills and HBSS was transferred to a 15mL tube and 2.5mL of prepared trypsin solution was added to the tube to obtain a final trypsin concentration of 0.05%. The tubes were then placed on a gyro-rocker and gently rocked for 10 minutes at room temperature (Figure 2.8b). After this, 5mL of fresh HBSS were added to each tube to

obtain the final volume of 10mL in order to dilute the enzyme. The contents of the tubes were then filtered through a 40µm cell strainer (Figure 2.9a) and the filtrate centrifuged for 15 minutes using a MSE Mistral 2000 with a speed of 3000rpm (Figure 2.9a). Finally, the supernatant was removed from each tube and the pellet was suspended in 0.5mL fresh HBSS, transferred to an Eppendorf tube and stored on ice or in the fridge for up to 24 hours for further stages.

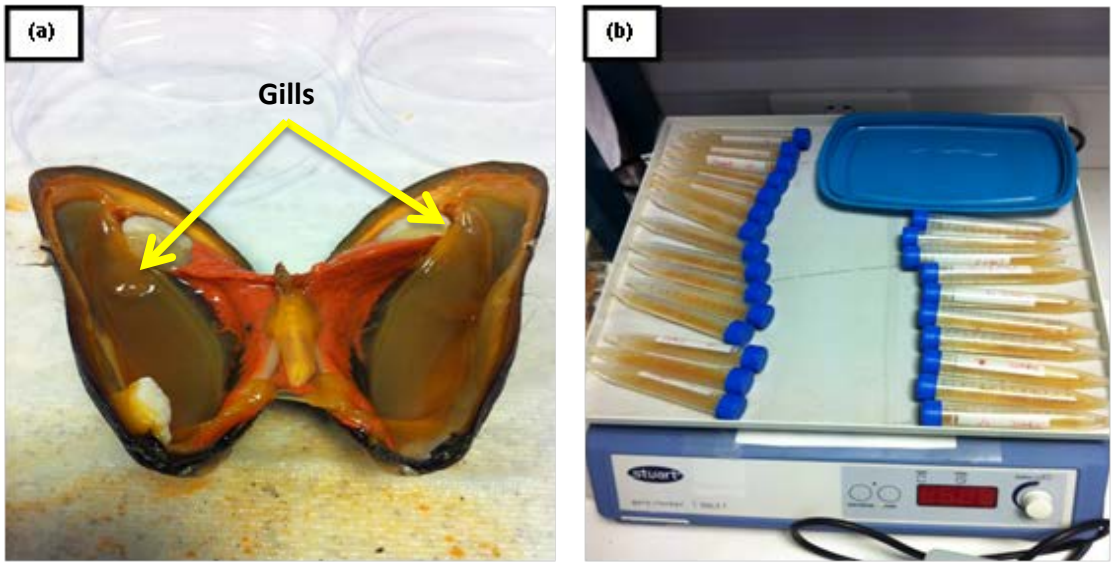


Figure 2.8: (a) The gill being extracted from both sides. (b) The gill tubes being rocked using a gyro-rocker.

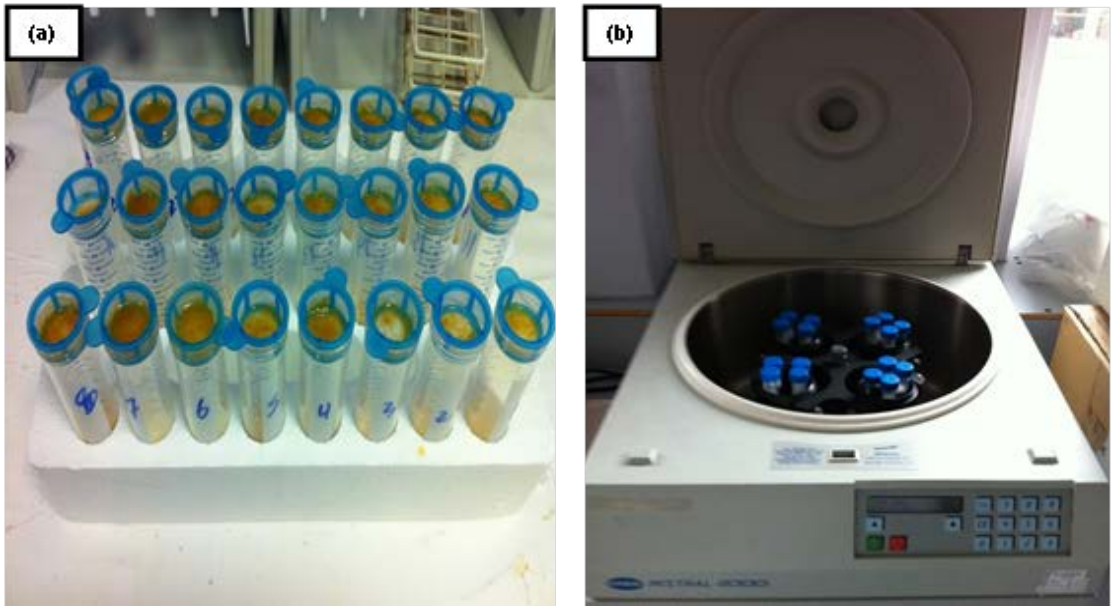


Figure 2.9: (a) The filtration process using 40 µm cell strainer. (d) Centrifuging the gill tubes using MSE Mistral 2000.

2.5 Characterization of CuO particles (NPs and MPs)

Copper oxide particles (CuO NPs and MPs) were characterised in order to establish particle/agglomerate/aggregate size and shape. Two different techniques were used in this study, transmission electron microscopy (TEM) and dynamic light scattering (DLS). In addition, the copper accumulation in mussel tissues were assessed using inductively coupled plasma optical emission spectrometry (ICPOES).

2.5.1 Transmission electron microscope (TEM)

Transmission electron microscopy (TEM) analysis was used to determine the shape and the size of CuO particles in the exposure medium, seawater (Figure 2.10a). Four different concentrations of each particle (5, 10, 15 and 20µgL^{-1}) were prepared separately (8 samples in total) and a droplet of each sample was placed on a Formvar/Carbon coated 200mesh Copper grid for 10 minutes. Excess liquid was removed by touching the edge of the grid with filter paper. The grid was left to air dry for 15 minutes (Figure 2.10b). The samples were then viewed in a Phillips CM120 transmission electron microscope (TEM, FEI UK Ltd, Cambridge, England) (Figure 10). Images were taken on a Gatan Orius CCD camera (Gatan UK, Oxon, England).

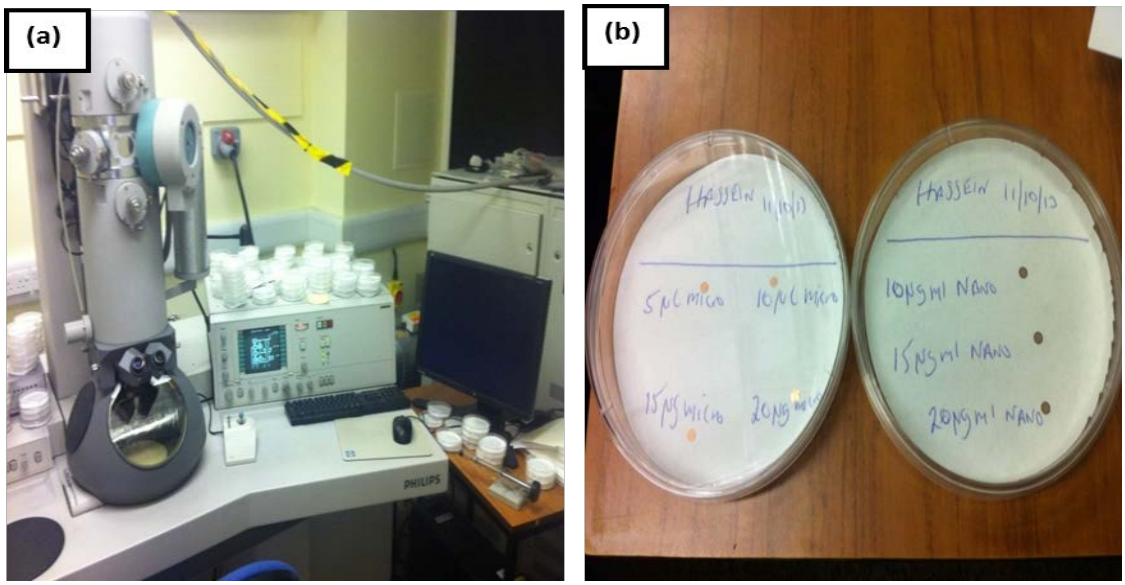


Figure 2.10: (a) Transmission Electron Microscope (TEM). (b) CuO particles (NPs and MPs) samples on the grid.

2.5.2 Dynamic Light Scattering (DLS)

The average agglomerates size and zeta potential of CuO particles (NPs and MPs) were measured using dynamic light scattering (DLS, Zeatsizer, nano series, and reference 2011143 from Malvern). In the DLS mode, four samples of each concentration of each particle (5, 10, 15 and 20µgL^{-1}) (8 samples in total) were prepared and 1mL of seawater (exposure medium). Each sample was then placed in a disposable capillary cuvette and placed on the device (Figure 2.11a). Measurements were set manually for 3 runs for both the size and the zeta potential for each sample at a controlled temperature of 25°C (Figure 2.11b).

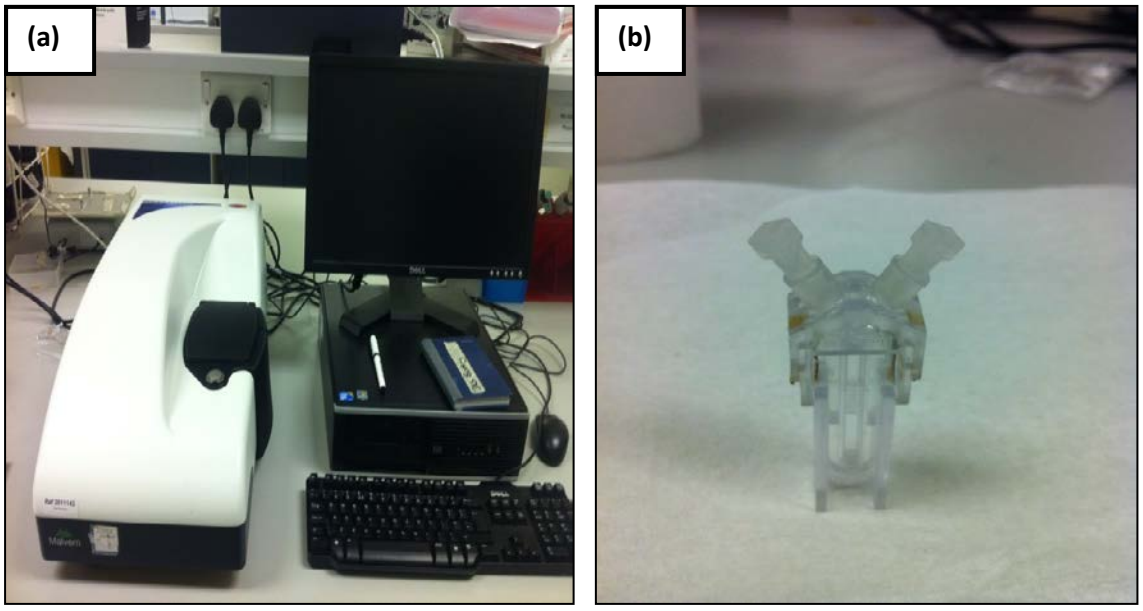


Figure 2.11: (a) Dynamic Light Scattering (Zeatsizer, nano series). (b) CuO particles (NPs and MPs) samples on the disposable capillary cuvette.

2.5.3 Copper analysis

Metal analysis was used to measure the total Cu content in medium (seawater) and accumulation of Cu in mussel tissues following exposure to different concentrations of the Cu forms using inductively coupled plasma optical emission spectrometry (ICPOES). For the accumulation of Cu in the mussel tissues, initially five empty clean glass beakers were prepared and weighed. Following 72 hours exposure, all the tissues were collected from each mussel and placed in separate pre-weighed beakers (5 mussel tissues in one beaker for each concentration) and the tissue wet weight

determined. Total Cu was extracted from the tissues by placing the beakers in an oven to dry at 60°C for at least 24 hours (Figure 2.12a) and then weighed again to obtain the tissue dry weight. Each beaker was topped up with 5mL of nitric acid (HNO_3) (S.G.1.42 (70%), from Fisher Scientific) and left for 24 hours (Figure 2.12b). Afterwards, 45mL of distilled water was added to each beaker which brought the total volume of 50mL and then placed on water bath at 60°C for 4 hours to extract Cu from the tissues (Figure 2.13a). The contents of the beakers were filtered using GF/C filter papers (Figure 2.13b), and then analysed using inductively coupled plasma optical emission spectrometry (ICPOES) (Perkin Elmer, Optima 5300 DV, and Reference (2083865)) (Figure 2.14) using a copper standard (Copper standard solution (1000 ppm), reference: J/802515, Batch: 0114095). Water samples were collected from each tank and put in 15mL tubes, and then all tubes were centrifuged for 10 minutes using a MSE Mistral 2000 with a speed of 3000 rpm. Samples were taken from supernatant and analysed directly by ICPOES.

Figure 2.12: (a) Drying the mussels' tissues in the oven at 60°C. (b) Nitric acid digestion to extract the Cu from tissues.

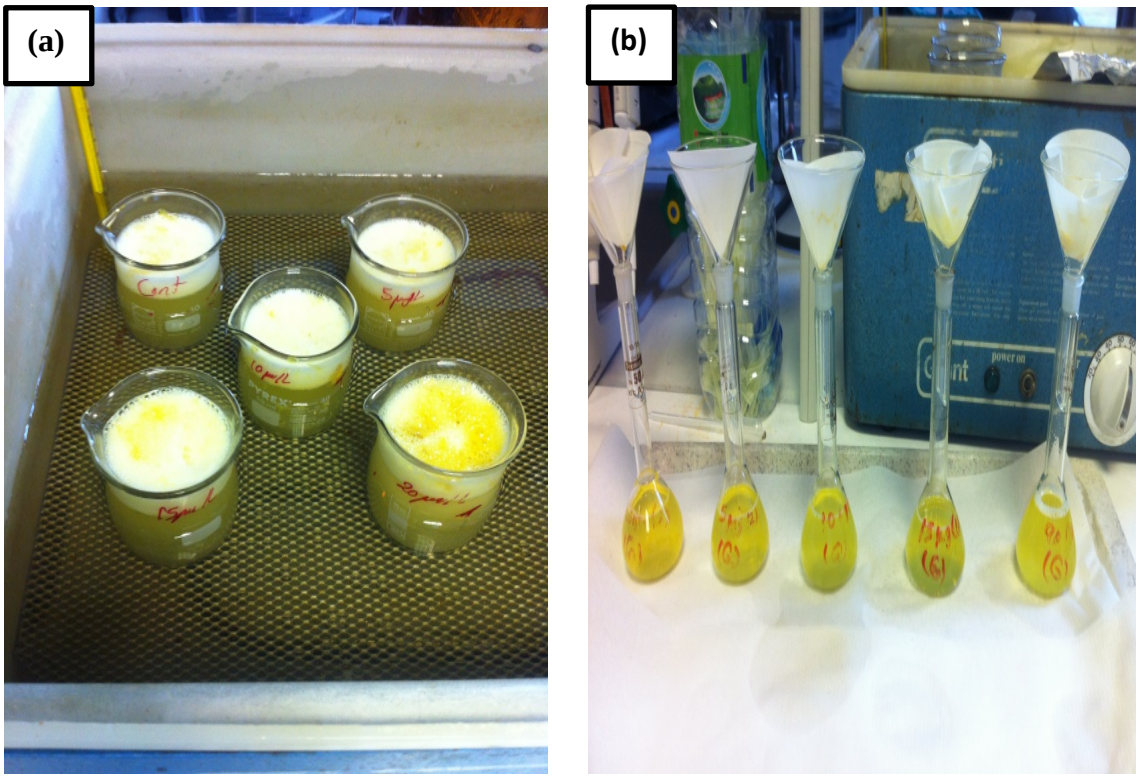


Figure 2.13: (a) Samples at water bath at 60°C. (b) Filtering the samples into 50 mL flasks using GF/C filter paper.

Figure 2.14: Inductively coupled plasma optical emission spectrometry (ICPOES) (Perkin Elmer, Optima 5300 DV, and Reference (2083865)).

2.6 Cell viability

The aim of this particular test was to assess the viability of the haemolymph cells of the mussels following exposure to different concentrations of different forms of Cu. Five tanks were prepared for the selected concentrations of each Cu form (5, 10, 15 and 20µgL^{-1}, along with the control (seawater only)). Three mussels with similar size (4.5 – 5.5 cm for *Mytilus edulis* and 9.5 – 10.5cm for *Modiolus modiolus*) were added to each tank which contained aerated seawater and an air pump, and left for 72 hours. All exposed mussels were taken out after the exposure period (72 hours), and haemolymph cells extracted as described above and kept in the fridge for 24 hours in order to assess the viability of the cells using techniques, trypan blue and flow cytometry (FC).

2.6.1 Trypan blue

Trypan blue is a simple and straightforward technique which is used to assess viability by staining the dead cells which can be seen and counted under the light microscope within 3 minutes. Based on Absolom (1986), a trypan blue assay was prepared by mixing 80µL of (0.2%) trypan blue dye with 20µL of (4.25%) sodium chloride (NaCl). Next, a mixture of 100µL of haemolymph cells and trypan blue stain was placed on a Neubauer Improved Haemocytometer and covered by a cover slip (22mm X 22mm), in order to count the cells under the normal light microscope (Figure 2.15 a and b).

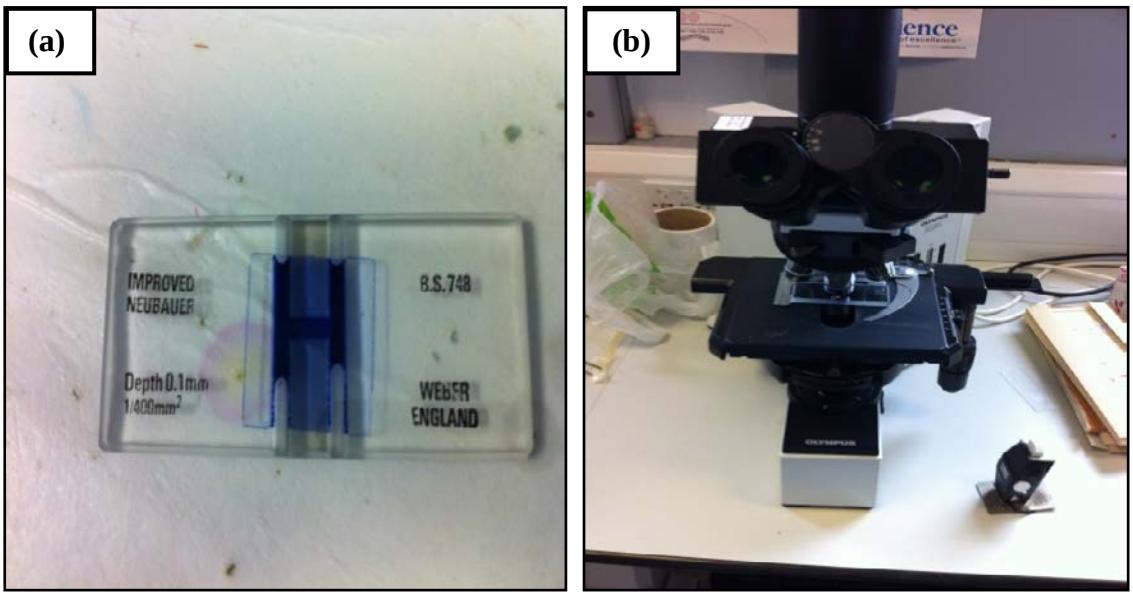


Figure 2.15: (a) The trypan blue slide. (b) The normal light microscope.

2.6.2 Flow cytometry

Flow cytometry is an effective technique due to its ability to count over 50,000 cells per second and that is used for identifying and counting cells in heterogeneous samples (Di Carlo et al., 2010). In this technique, two samples of haemocytes of each concentration were prepared each of which were made by taking 200µL of haemolymph and mixing it with 800µL of HBSS solution (HBSS has no NaCl to reduce the background that generated from salt, which impact of the result profile) in tubes (Röhren tubes 3.5mL, 55X 12mm) (Figure 2.16a) which fit into the flow cytometry device and kept on ice. Next, all samples were stained with propidium iodide (Pi) stain which was prepared by diluting 100µL of Pi in 1mL distilled water, and stored in the dark (Pi is sensitive to ultraviolet light) until use. All the samples were then analysed by flow cytometry (FC) (A Partec CyFlow reference 2005442-1711) (Figure 2.16b).

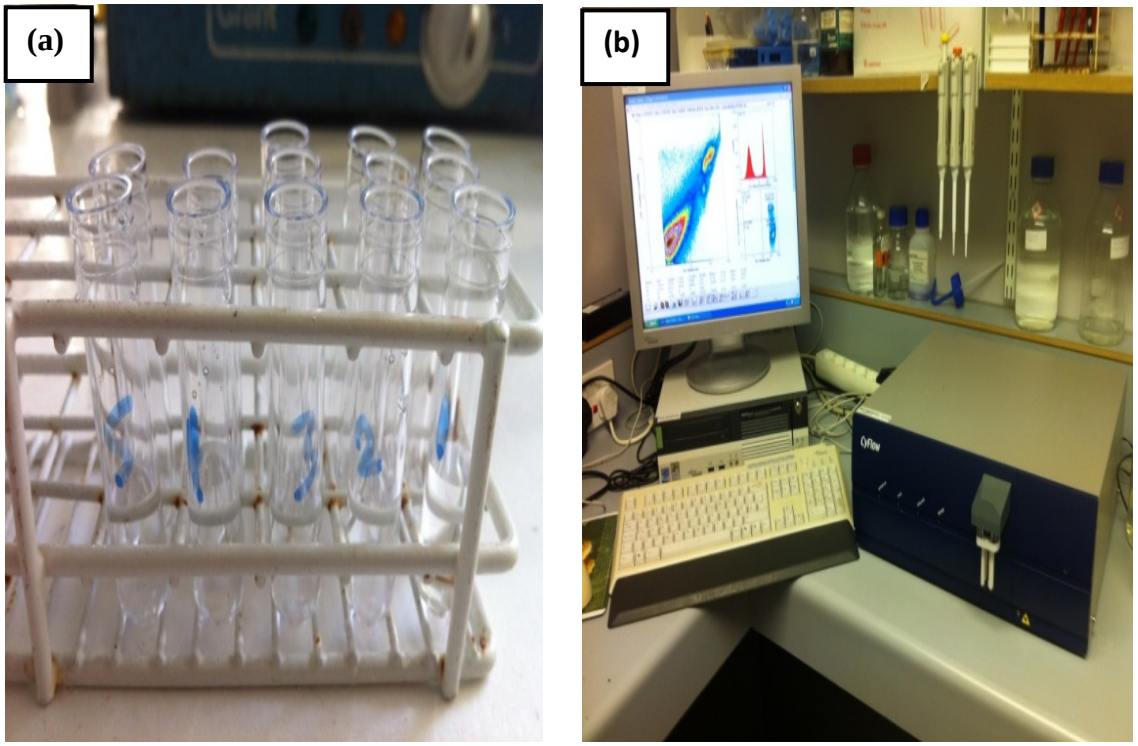


Figure 2.16: (a) The RÖhren tubes (3.5ml, 55X 12mm). (b) The Flow cytometry (FC) (A Partec CyFlow reference 2005442-1711).

2.7 Single cell gel electrophoresis (SCGE) or Comet assay

2.7.1 Chemical solution preparation

Before carrying out the SCGE procedure, several chemical solutions were prepared 24 hours before starting the experiment. All the chemical stocks were obtained from the commercial source Sigma- Aldrich, apart from tris hydrochloride (Tris) stock solution which was obtained from VWR international Ltd.

All the solutions described below were prepared according to Hartl et al. (2010)

- **Phosphate buffered saline (PBS)**

PBS was prepared by dissolving 1 tablet of PBS in 200mL of distilled water. The mixture was placed on a strainer for at least 5 minutes for complete dissolution, and stored at room temperature.

- **Normal gel agarose (NGA)**

NGA was prepared by dissolving 1g of NGA powder in 100mL of prepared PBS by heating the mixture to obtain complete dissolution (clean solution) and then stored in the fridge at 4°C.

- **Low melting agarose (LMP)**

This was prepared in the same way as NGA by dissolving 1g of LMP powder in 100mL of prepared PBS by heating the mixture on lower heat than for NGA to avoid reaching boiling point, and then stored in the fridge at 4°C.

- **Sodium hydroxide (NaOH)**

10N NaOH was prepared by dissolving 400g of NaOH powder in 1000mL of distilled water, and then stored at room temperature.

- **Ethylenediaminetetraacetic acid (EDTA)**

EDTA (200mM) was prepared by dissolving 23.37g of EDTA powder in 200mL distilled water, then NaOH was added gradually to obtained a pH of 7 and then another

200mL of distilled water was added to the contents to make the final volume of 400mL and stored at room temperature.

- **Tris hydrochloride (Tris)**

Tris (0.4mM) was prepared by dissolving 31.52g of Tris powder in 500mL of distilled water and stored in the fridge at 4°C.

- **Lysing solution stock**

The Lysing solution was prepared as follows: 48.4g of NaCl, 44g of EDTA, 2.36g of Tris and 15g of N-Lauroylsarcosine sodium salt were dissolved in 1000mL of distilled water. NaOH was then added gradually to obtain pH of 10 and then the solution was made up to 1500mL and stored in the fridge at 4°C.

- **Lysis working solution**

Lysis working solution was prepared on the day using a small plastic slide developing box (Figure 18b). 1.5mL of Triton X-100 was added to 135mL of prepared lysing solution, followed by the addition of 15mL dimethyl sulphoxide (DMSO) and then stored in the fridge at 4°C until use.

- **Electrophoresis solution**

Electrophoresis solution was prepared on the day by adding 60mL of pre-prepared 10N NaOH solution and 10mL of 200mM EDTA solution, to chilled distilled water (final volume: 2L), repeatedly inverted to ensure mixing and stored in the fridge at 4°C until use.

- **Gelred stain**

The Gelred stain was prepared by diluting 2µL of gelred dye in 10mL of distilled water and kept in a dark area at room temperature.

- **Hanks buffer saline solution (HBSS)**

HBSS was obtained and adjusted for the mussel's haemolymph osmolality by adding 11.1gL^{-1} of sodium chloride (NaCl) to 500mL of HBSS solution and stored in the fridge at 4°C.

2.7.2 Comet assay or SCGE procedure

The comet assay involves many steps. These comprise embedding nucleated single cells into an agarose sandwich on a microscope slide, removing the cell membranes, an electrophoresis stage, and neutralisation and staining of the cells and viewing under a Zeiss Axiophot microscope (Figure 2.17).

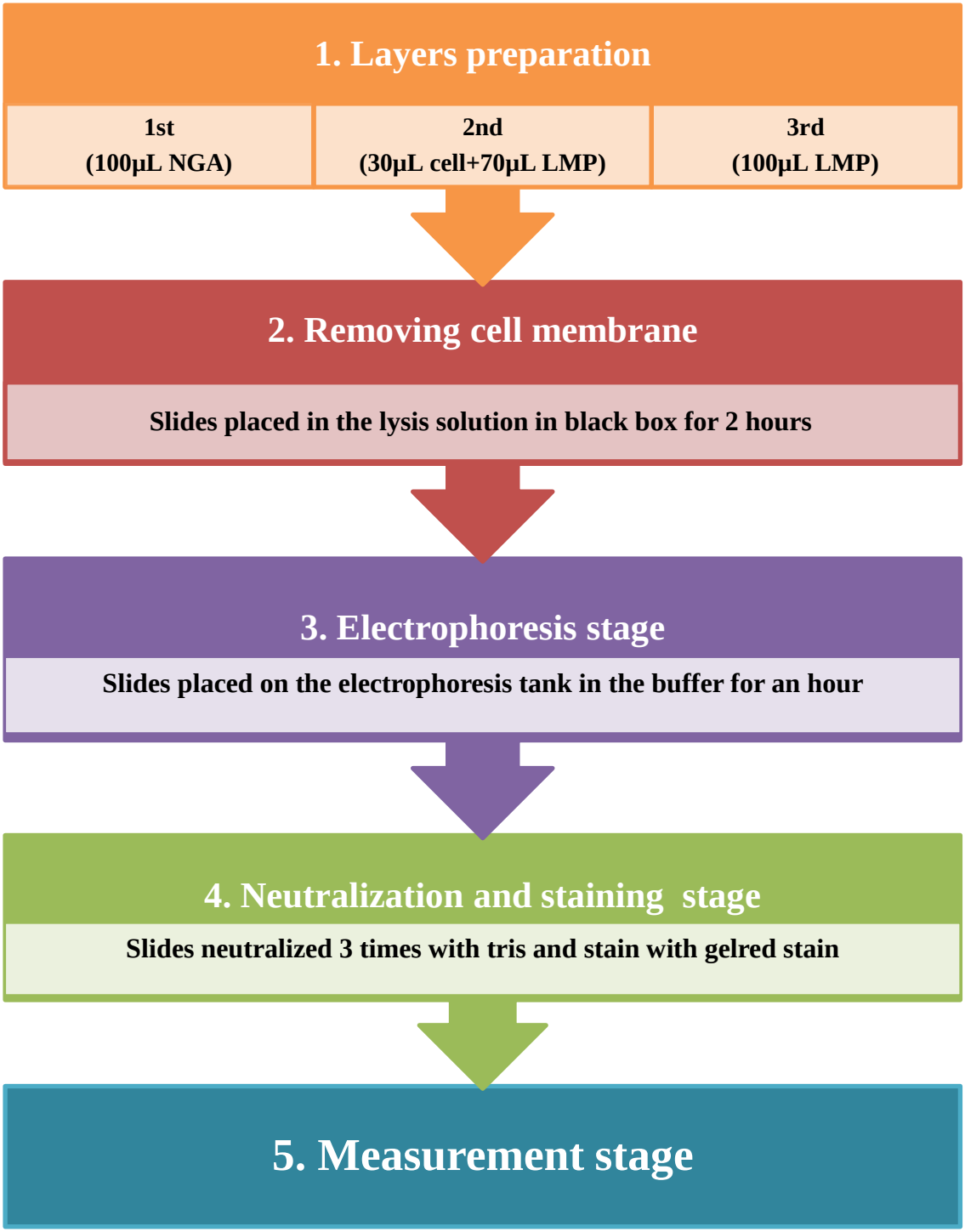


Figure 2.17: A summary of the Comet assay procedure.

(1) Preparation of slides

Fifty microscope slides (25 slides for each cell type) were prepared ("frosted") by applying 1% of melting normal gel agarose (NGA) and spreading it evenly across the slides, the slides were then marked with a pencil and allowed to dry for 24 hours. In order to produce the minigels, the slides were placed on the hot plate at 40°C, to be warmed and help to spread the gel evenly underneath the cover slips, avoiding any bubbles. The first layer was prepared by placing 100µL of melted NGA on each frosted marked slide, applying a cover slip (22mm X 22mm) leaving at 4°C for 20 minutes to set. Next, the slides were taken out and the cover slips were gently removed; then a mixture of 30µL of cell suspension and 70µL low melting agarose (LMP) was placed on top of the first layer of each slide, cover slip quickly applied and left at 4°C for 20 minutes to set. Finally, the slides were taken out and the cover slips removed, then 100µL melting low melting agarose (LMP) added on top of the second layer of each slide, a coverslip quickly applied and stored at 4°C for another 20 minutes (Figure 2.18a).

(2) Removal of cell membranes

After the agarose had solidified the cell, nuclear and cell membranes were removed by taking the slides from the fridge, removing the cover slips and placing the slides, in the lysis solution in light-proof black box (Figure 2.18b) and stored in the fridge at 4°C for at least 2 hours.

(3) Electrophoresis stage

The electrophoresis tank (Model A3-1, Rated 0-250V, 0-100mA, Serial No.233712 from Owl Separation Systems, Inc.) was prepared and slides were placed horizontally with the same orientation (left to right) in the tank. Then pre-prepared electrophoresis solution was poured slowly from the right side of the tank until it covered all the slides and left for 30 minutes. The reason for this process is to denature and unwind the DNA. After 30 minutes, the power (25V and 300mA) was applied for another 25 minutes, without changing the electrophoresis solution (Figure 2.19a).

(4) Neutralisation and staining

The slides were gently removed from the electrophoresis tank and the gels neutralised three times with 5 drops of a pre-prepared tris solution, with 5 minutes between each addition. Then the slides were rinsed with 5 drops of chilled water, excess water removed and the gels stained with 5 drops of pre-prepared Gelred, cover slips applied and the slides kept in the fridge overnight at 4°C (Figure 2.19b).

(5) DNA measurement under the microscope

The slides were observed under a Zeiss Axiophot microscope using 400x magnifications. Fifty randomly selected cells per slide were scored using the software package Comet Assay IV (Perceptive Instruments) and the DNA damage was expressed as the percentage of DNA in the tail (Figure 2.20 a and b).

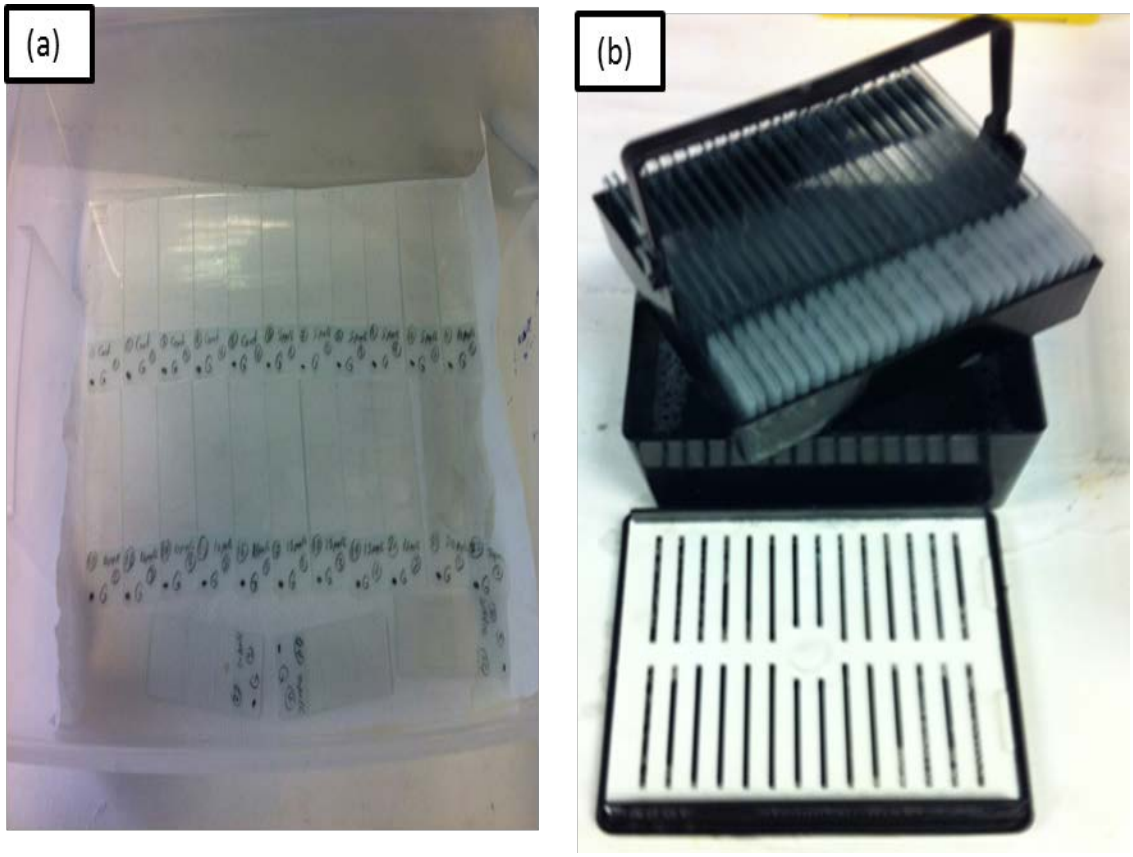


Figure 2.18: (a) Layers preparation. (b) Removing cell membranes with Lysis solution.

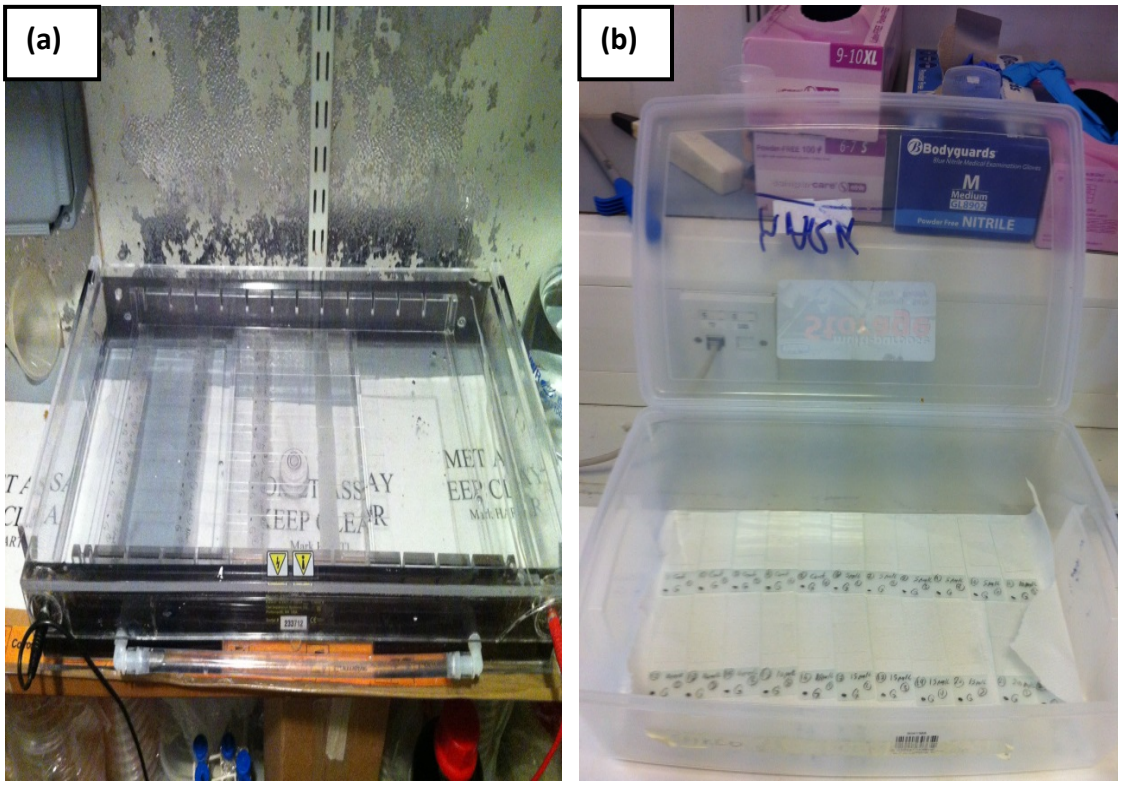


Figure 2.19: (a) Electrophoresis stage. (b) Neutralisation and staining the DNA.

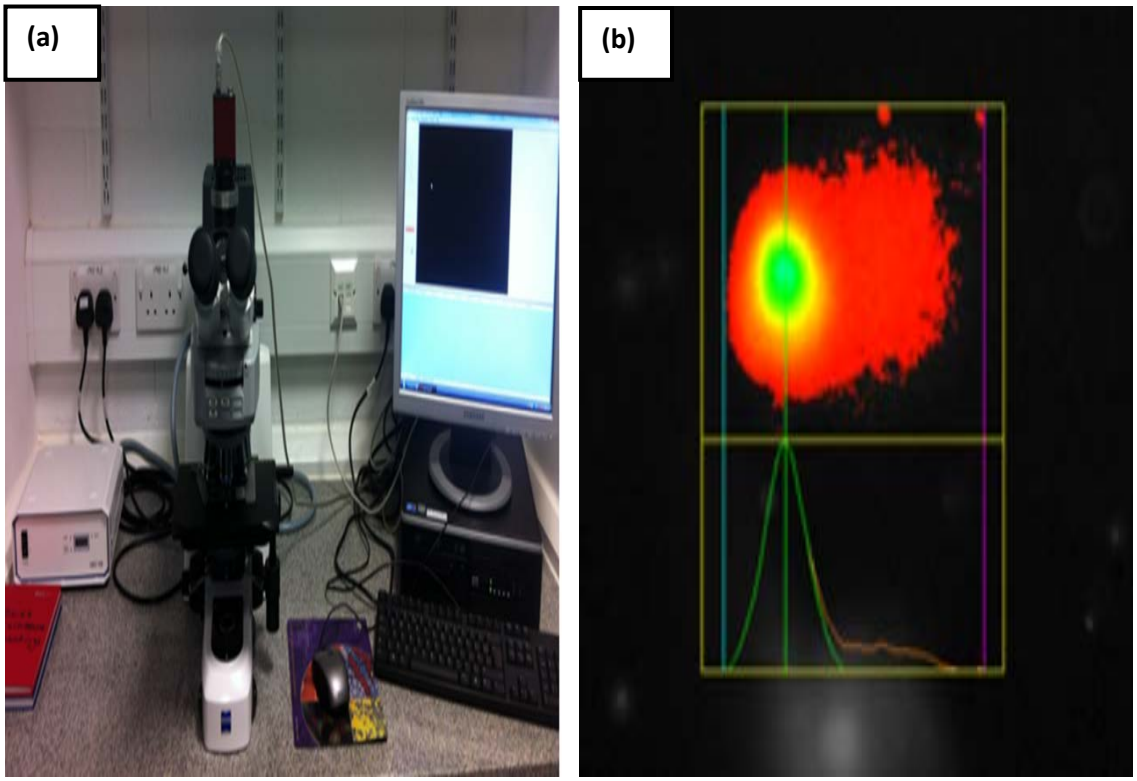


Figure 2.20: (a) Zeiss Axiophot Microscope. (b) DNA damage image.

2.8 Superoxide dismutase (SOD) assay

The superoxide dismutase (SOD) assay was used in this study to measure the oxidative stress by detecting the activity of SOD (expressed in % inhibition) in the mussels following exposure to different concentrations of the three forms of Cu. In the present study, a SOD kit was obtained from Sigma-Aldrich (Reference: 19160-1KT-F). The kit contains 5mL of working solution (WST), 100µL of enzyme working solution, 100mL of buffer solution and 50mL of dilution buffer (Figure 2.21).

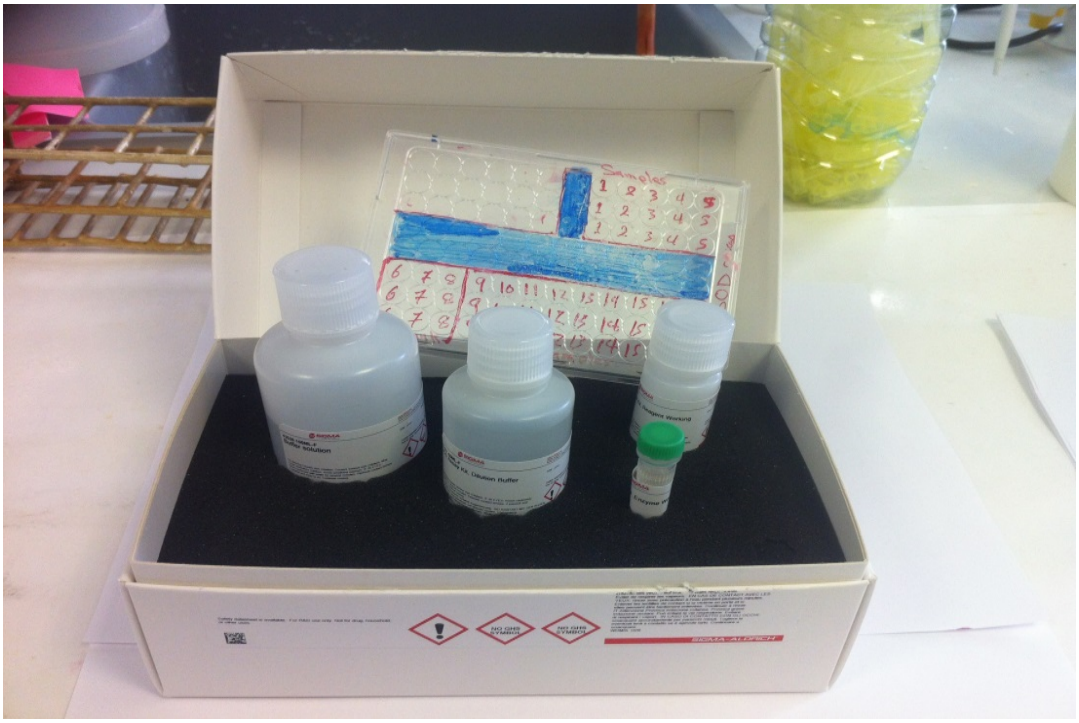


Figure 2.21: The SOD kit (WST solution, enzyme working solution, buffer solution and dilution buffer).

2.8.1 Regent preparation

Before starting the SOD measurement, several reagents or solution require preparation as per kit instruction. This are described below.

- **Working solution (WST):** WST was prepared by diluting 1mL of WST solution in 19mL of buffer solution and then store in the fridge.

- **Enzyme working solution:** The enzyme working solution was prepared by centrifuging the enzyme solution for 5 seconds, mixing by pipetting, and then diluting 15µL of enzyme solution with 2.5mL of dilution buffer, and stored in the fridge

2.8.2 SOD assay protocol

At the early stage following the exposure period, the gill samples were extracted and dipped in liquid nitrogen and stored in the freezer at -80°C. Then all the samples were taken out and placed into a glass homogenizing tube and carefully homogenized in 1:5 vol. of homogenizing buffer (Tris- HCl 50mM, 0.15M KCl. pH 7.4) with a Teflon pestle. The homogenate was split and transferred into two 1mL Eppendorf tubes (one tube was stored at -20 °C for TBARS and total protein assays and the other one was used for SOD assay). SOD samples were prepared according to the protocol reported by Almeida et al. (2005). All SOD samples were centrifuged at 13.000 x g for 30 min at 4°C. Then, the supernatant fractions were transferred into 15mL Beckmann ultracentrifuge tubes and centrifuged at 40,000 x g for 60 minutes at 4°C in order to obtain the cytosolic fraction. After that, a 96-well microplate was prepared as illustrated in the table below, using the samples and the SOD reagents (Figure 2.22).

Table 2.1: The amount of each solution for each sample, Blank 1, 2 and 3

	Samples	**Blank 1**	**Blank 2**	**Blank 3**
Sample solution	**20µL**	**-**	**20µL**	**-**
DDH_2O	**-**	**20µL**	**-**	**20µL**
WST working solution	**200µL**	**200µL**	**200µL**	**200µL**
Enzyme working solution	**20µL**	**20µL**	**-**	**-**
Dilution buffer	**-**	**-**	**20µL**	**20µL**

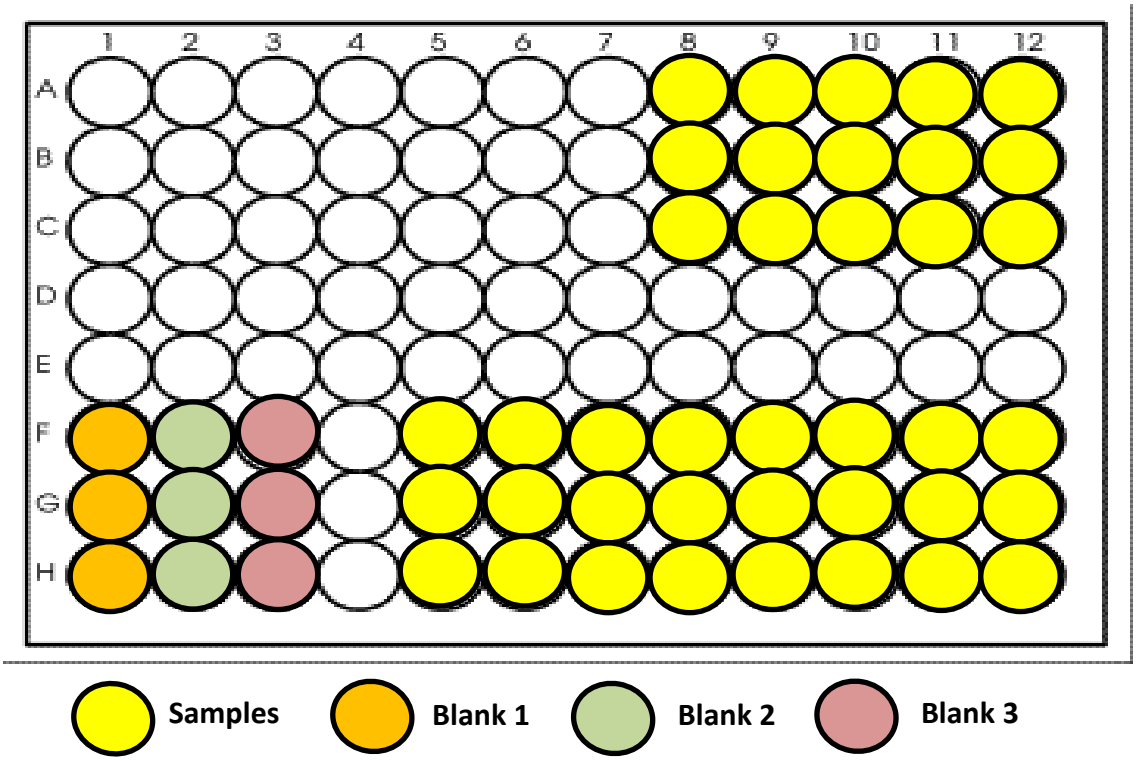


Figure 2.22: A prepared 96-well microplate including samples and reagents.

After that, the 96-well microplate was incubated at 30°C for 30 minutes and read at 450 595nm using a spectra Max M5 microplate reader (Figure 2.23 a and b). Finally, the values were analysed as % inhibition of SOD activity and were calculated using the equation below.

SOD activity (inhibition rate %) =

$$\left[\frac{(Blank_1 - Blank_3) - (Sample - Blank_2)}{(Blank_1 - Blank_3)} X\ 100\right] \qquad \textbf{(2)}$$

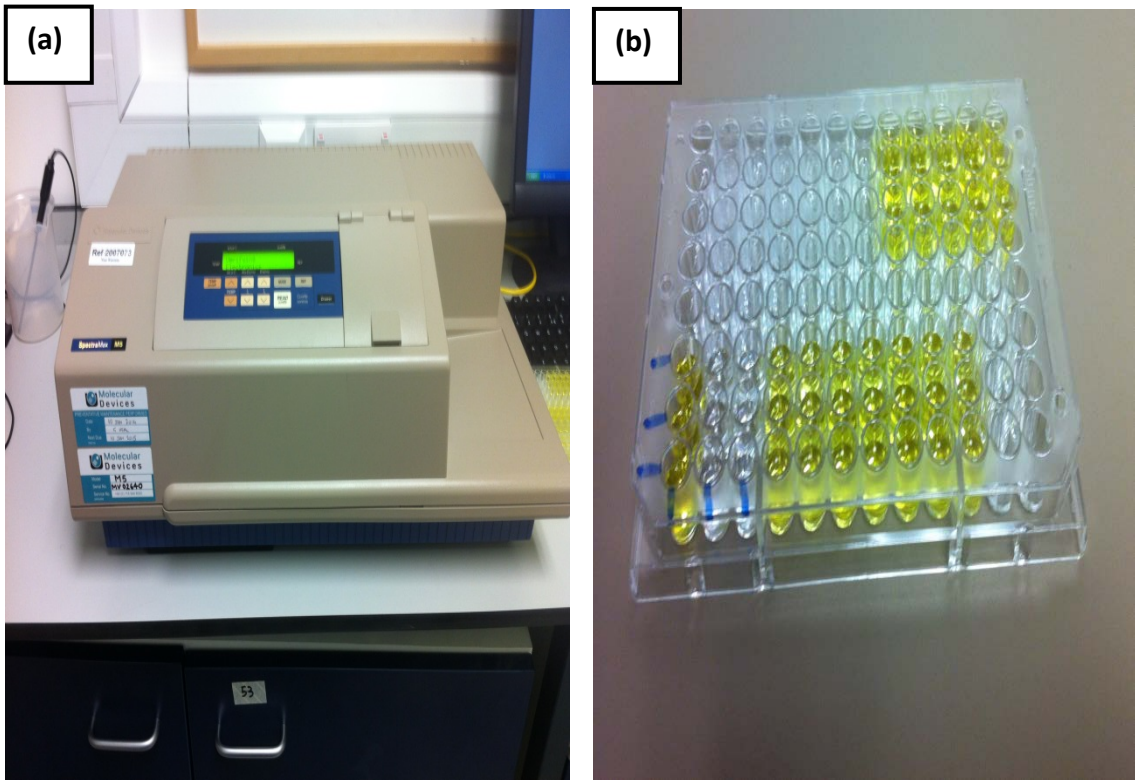


Figure 2.23: (a) The microplate reader (spectra Max M5). (b) A prepared 96-well microplate after 30 min of incubation.

2.9 Thiobarbituric acid reactive substances (TBARS) assay

The thiobarbituric acid (TBA) or TBARS assay was used in this study to evaluate the free radical-mediated oxidation of biological systems that include polyunsaturated fatty acids (PUFA) found in cell membranes (Camejo. et al., 2007).

2.9.1 Regents preparation

Several reagents, such as BHT, PBS, TCA and TBA, were prepared and stored at room temperature 24 hours before carrying out the experiment, as reported by Camejo et al. (2007) (Figure 2.24). These are indicated below.

- **Butylated hydroxytoluene (BHT) solution:**

BHT solution was prepared by dissolving 23.3g of butylated hydroxytoluene (2, 6-Di-O-tert-butyl-4-methylphenol; Reference: 058K0134) in 100mL absolute ethanol.

- **Phosphate buffered saline (PBS):**

PBS solution was prepared by diluting 58.45g of EDTA in 200mL distilled water, the pH adjusted to 7.4 and 1 tablet of Phosphate buffered saline (Reference:P4417-100TAB).

- **Trichloroacetic acid (TCA) solution:**

TCA solution was prepared by dissolving 50g of trichloroacetic acid (Reference: T6399-250G) in 100mL distilled water.

- **Thiobarbituric acid (TBA) solution:**

TBA solution was prepared by diluting 0.3g of Sodium hydroxide (NaOH) pellets in 100mL distilled water. Then, 1.3g of thiobarbituric acid (Reference: S42774-308) was dissolved in 100mL of prepared NaOH solution

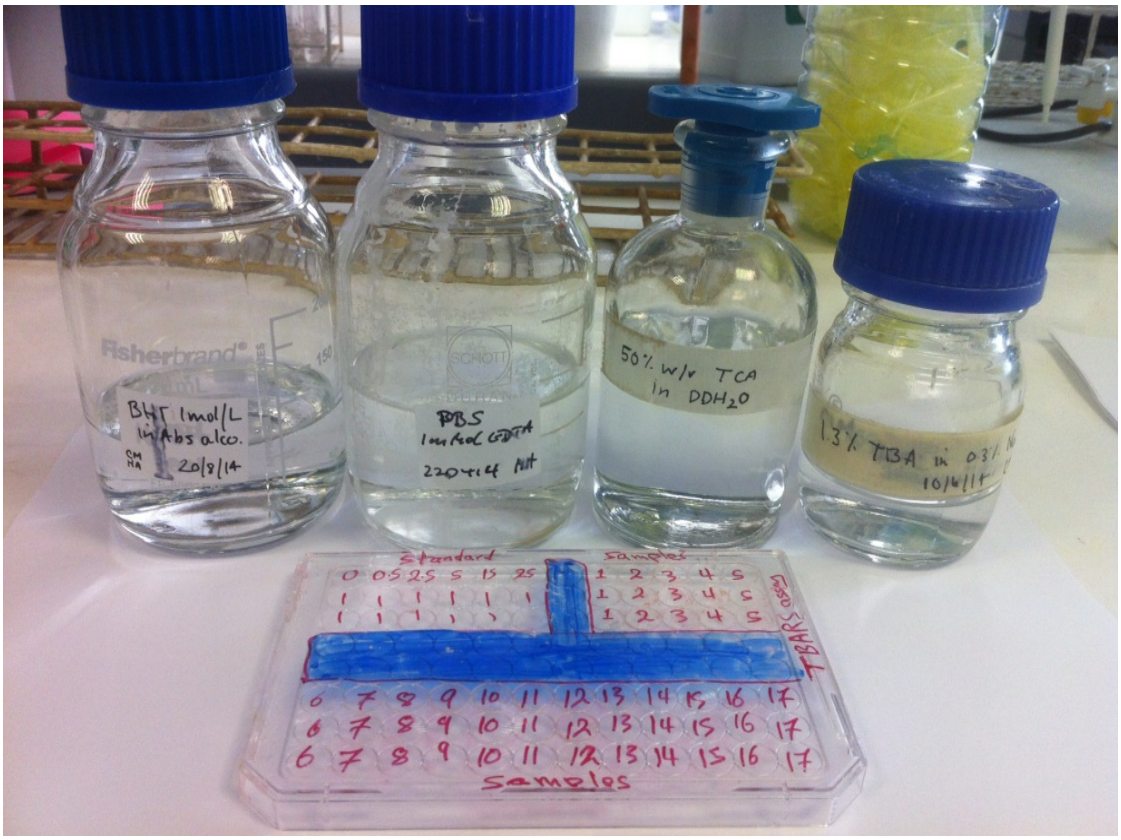


Figure 2.24: TBARS assay reagents solution and 96-well microplate layouts.

2.9.2 TBARS assay protocol

All the frozen gill samples were thawed and centrifuged at 13.000 x g for 5 min at 4 °C. Then, the TBARS standards (0, 0.5, 2.5, 5, 15 and 25pMol TEP) were prepared as described below (Table 2.2). A 96-well microplate was prepared by adding 40µL of each gill samples in triplicate to each well. The same was done for TEP standards. After that, all prepared reagents were added as follows: 10µL of BHT, 140µL of PBS, 50µL of TCA and 75µL of TBA, and the plate incubated at 60°C for 60 minutes (Figure 2.25a).

Table 2.2: TBARS standards preparation calculation.

Step	TEP (µL)		Ethanol (µL)	Concentrations (nMol)
1	100		10,000	1,000,000
2	10 of 1	+	4000	25
3	600 of 2		996	15
4	400 of 3		1,200	5
5	400 of 4		800	2.5
6	300 of 5		1,500	0.5

During the incubation period time, the total protein concentrations of all samples were measured in a separate microtiter plate. This process was carried out according to Bradford (1976). Firstly, Bovine Serum Albumin (BSA) standards were prepared (1, 0.8, 0.6, 0.4, 0.2, 0mgmL^{-1}) and 10µL of each standard pipetted in triplicate wells. Secondly, 10µL of each sample was diluted in 900µL of SOD homogenizing buffer and then 10µL was pipetted into 3 replicate wells. Finally, 290µL of Bradford reagent was added to each well including standard wells, the plate incubated in the dark for 5 minutes. Following, and read at 595nm using a microplate reader (spectra Max M5) (Figure 2.25b). The TBARS plates were read using the spectra Max M5 at 530nm and

then at 630nm to correct for turbidity. Finally, TBARS were calculated using the following equations:

$$Abs = A^{530} - A^{630} \quad (3)$$

$$TBARS = \frac{Abs}{Protein(Sample\ x\ 10)}\ nmol\ mg\ protein^{-1} \quad (4)$$

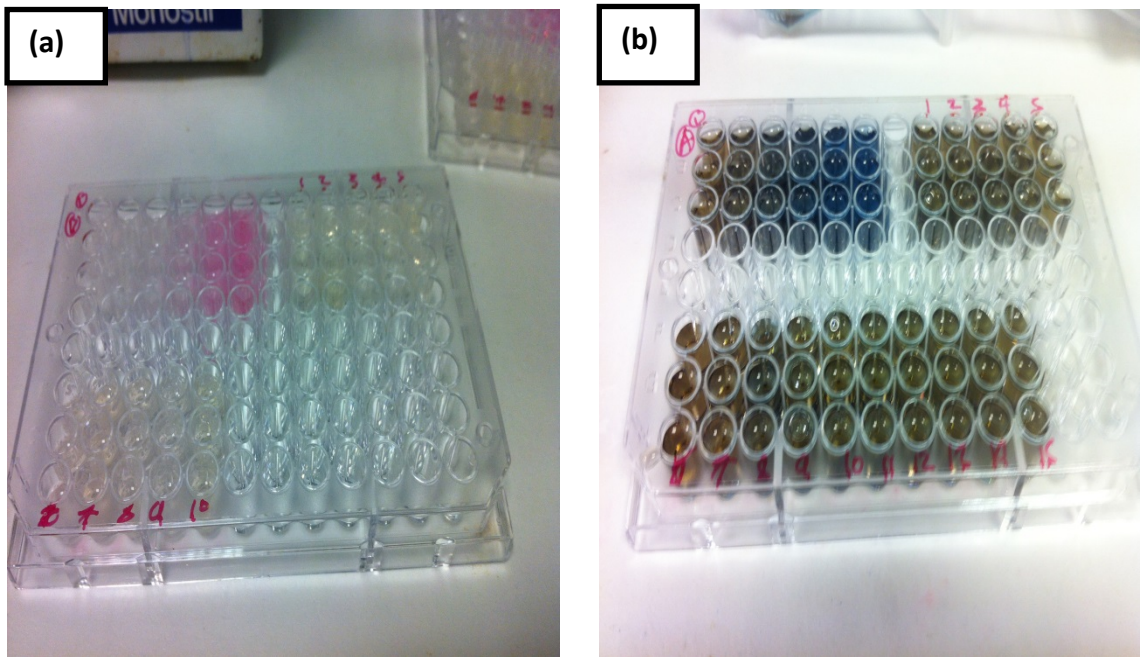


Figure 2.25: (a) Prepared TBARS plate after incubation period 60 minutes. (b) Prepared BSA plate.

2.10 Statistical analyses

All data obtained from the study were tested for normality and homogeneity of variances. Then, statistical analysis was carried out using Sigmastat 2.03. If data were found to comply with parametric tests requirements, an analysis of variance (ANOVA) test, followed by the Tukey test, was used in order to determine any significant differences between treatment groups in each form of Cu as well as between NPs and other forms of Cu (MPs and salt). Statistical significance was set at $P<0.05$.

3 RESULTS

This section describes the outcomes of all experiments conducted in this study. A detailed description of the characterization of Cu particles (NPs and MPs) obtained by different techniques, including transmission electron microscope (TEM) and dynamic light scattering (DLS), will be illustrated. Then, all the assay results for each mussel species are presented, separately, starting with *M. edulis,* followed by *M. modiolus.* First, the concentration of Cu in both exposure medium (seawater) and mussel tissue, determined by the inductively coupled plasma mass spectrometry (ICP-MS), are presented, followed by the results of cell viability measurements of haemolymph cells of *M. edilus,* comparing both flow cytometry and trypan blue approaches. This is followed by genotoxicity and oxidative stress results (SOD activity and lipid peroxidation) of both *M. edilus* and *M. modiolus*. Finally, the results of the toxicity of different forms of Cu of both mussels (*M. edilus* and *M. modiolus)* will be described and compared.

3.1 Characterization of CuO particles (NPs and MPs)

3.1.1 Transmission electron microscope (TEM)

The particle size and the shape of both forms of particulate CuO (NPs and MPs) were analysed in the stock suspension using transmission electron microscope (TEM). Both particles forms of CuO (NPs and MPs) were successfully observed under TEM. To my knowledge this is the first reported instance of TEM sample preparation in NMs seawater exposure (Figure 3.1 and Figure 3.2). The particle sizes obtained by TEM of CuO NPs and MPs and suspended in seawater agreed approximately with the size specifications of the manufacturer for their respective powder forms (less than 50nm and less than 5µm, respectively). The CuO NPs suspended in seawater were observed to be spherical in shape and tended to agglomerate in the medium (Figure 3.1), whilst the CuO MPs were reticular particles and appeared less aggregated (Figure 3.2).

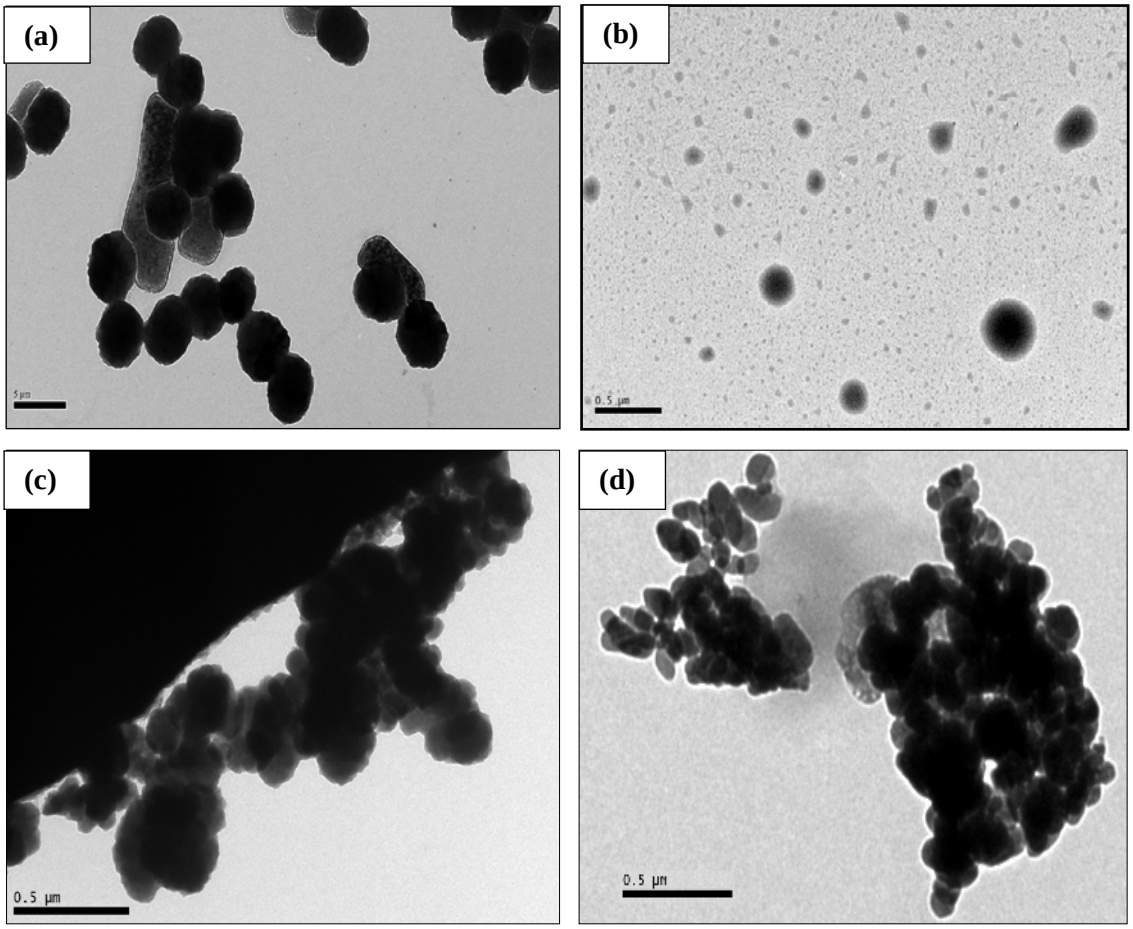


Figure 3.1: Transmission electron microscope (TEM) images of different concentrations of CuO NPs in seawater medium at scale bar 0.5µm. (a) $5\mu gL^{-1}$. (b) $10\mu gL^{-1}$. (c) $15\mu gL^{-1}$. (d) $20\mu gL^{-1}$.

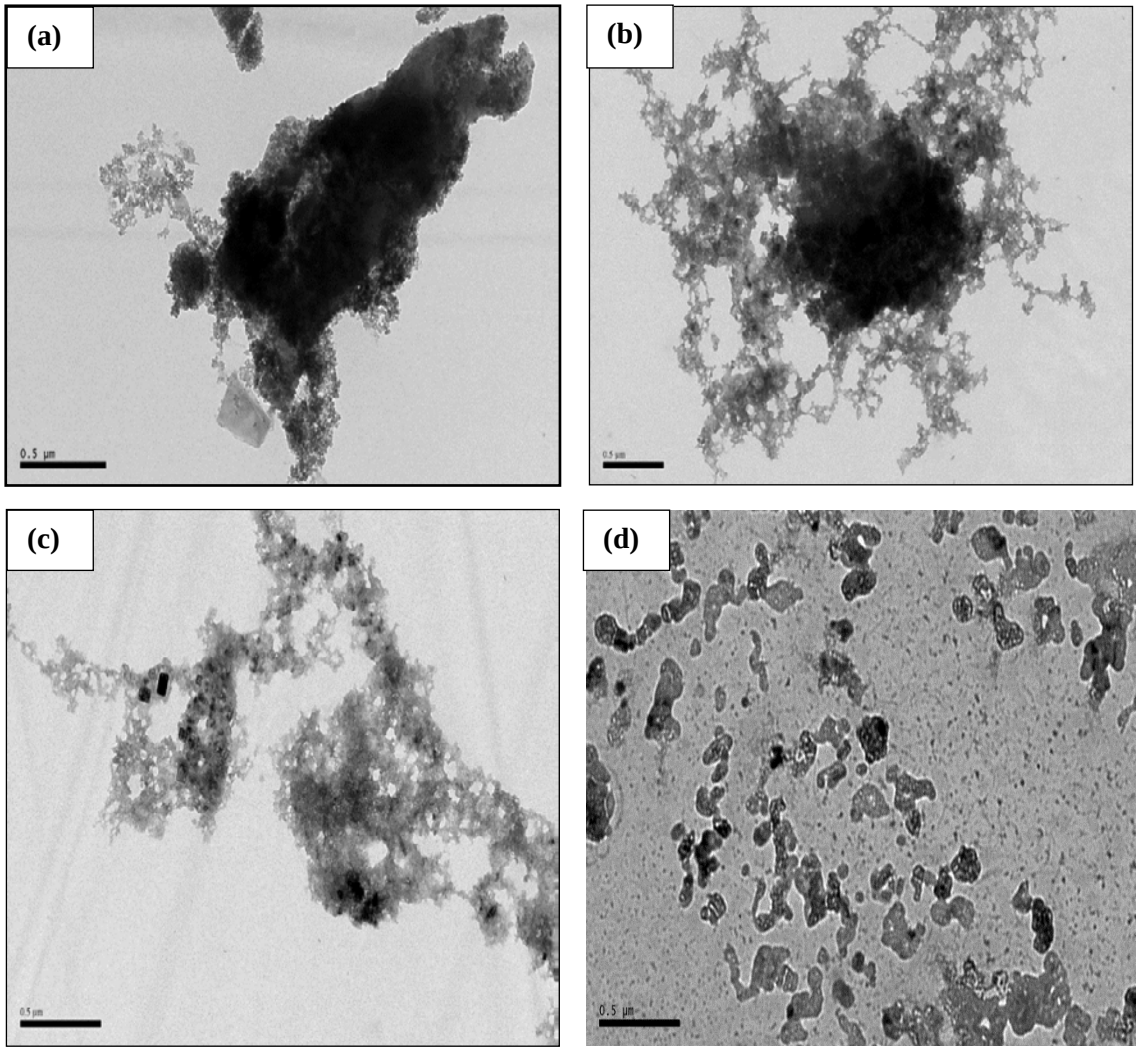


Figure 3.2: Transmission electron microscope (TEM) images of different concentrations of CuO MPs in seawater medium at scale bar 0.5µm. (a) 5µgL^{-1}. (b) 10µgL^{-1}. (c) 15µgL^{-1}. (d) 20µgL^{-1}.

3.1.2 Dynamic light scattering (DLS)

The average aggregation size and zeta potential of both forms of particulate CuO (NPs and MPs) were measured by dynamic light scattering (DLS) (Figure 3.3 and Table 3.1). The DLS analysis results showed that both types of CuO particles aggregated in seawater and the aggregation size average increased gradually as the concentration of CuO increased (reached 1018.2nm and 938.2nm at the highest concentration 20µgL^{-1} for CuO NPs and CuO MPs, respectively) (Figure 3.3 and Table 3.1). The zeta potential of the particles remained negative at all concentrations for both forms of particulate CuO (NPs and MPs) and the value ranged between -2.25 and -7.41mV and -2.20 and -11.51mV, respectively (Table 3.1).

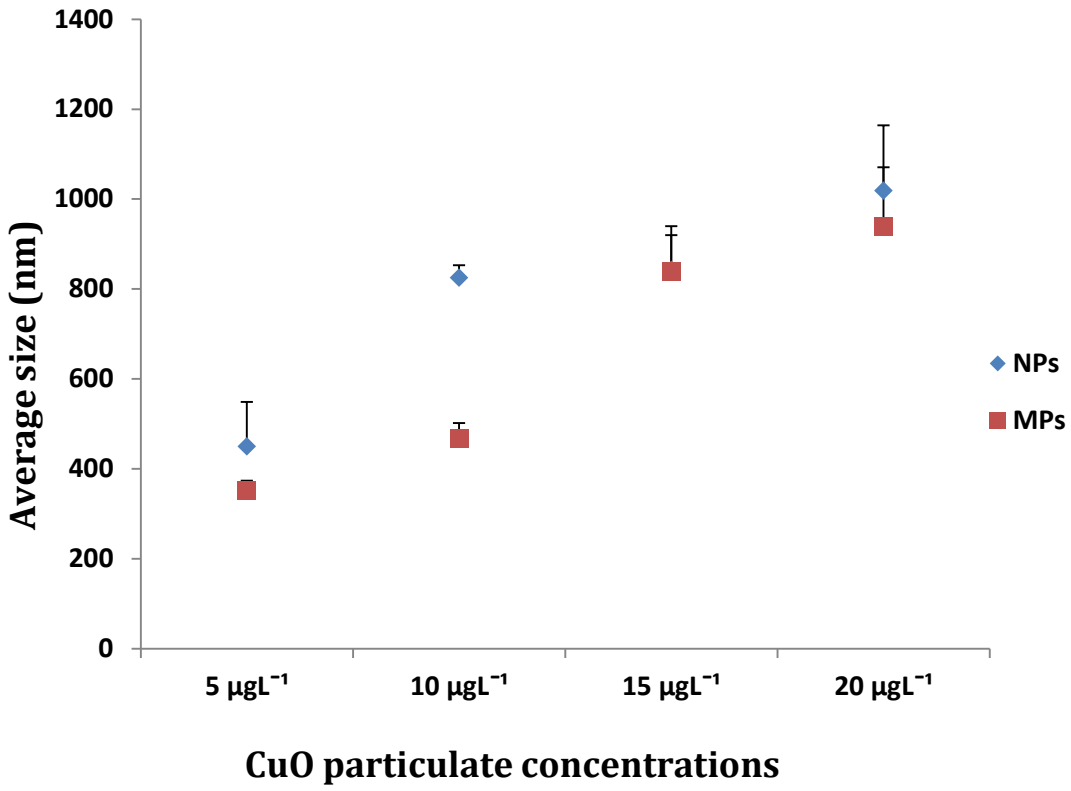


Figure 3.3: Average aggregated size of Cu particulate (NPs and MPs) at nominal concentrations in seawater obtained by dynamic light scattering (DLS) (n=3) (means ± standard deviation: only one arm shown) (note: at 15µgL^{-1}, there is no difference in the aggregate size value, so MPs value (red square shape) is overlap with the NPs value (blue diamond shape).

Table 3.1: Average aggregate size and zeta potential of CuO particulate (NPs and MPs) at different concentrations suspended in seawater medium measured by DLS.

	CuO NPs		**CuO MPs**	
CuO Concentration	**Aggregate average**	**Zeta potential average**	**Aggregate average**	**Zeta potential Average**
5µgL^{-1}	**449.2nm**	**-5.68mV**	**352.5nm**	**-11.51mV**
10µgL^{-1}	**824.7nm**	**-5.25mV**	**468nm**	**-2.24mV**
15µgL^{-1}	**841.7nm**	**-7.41mV**	**839.4nm**	**-8.91mV**
20µgL^{-1}	**1018.2nm**	**-2.25mV**	**938.2nm**	**-2.20mV**

3.2 *M. edulis* mussels

3.2.1 Cu concentrations

The total Cu concentration in both exposure medium (seawater) and *M. edilus* tissues was analysed after separate exposure to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at the nominal concentrations of 5, 10, 15 and 20μgL^{-1} for 72 hours, using inductively coupled plasma optical emission spectrometry (ICPOES) (Figure 2.14). Results indicated overall higher copper in the medium of the NPs and salt exposures when compared with the MPs (apart from at 20μgL^{-1}) (Figure 3.4A). In contrast, there were consistently higher concentrations of copper in the flesh of the mussels in the copper salt exposures when compared to the particulate exposures (NPs and MPs), (especially at 10μgL^{-1}, which could be probably due to high uptake rate by mussels in this concentration, although this was not fully tested in this study) (Figure 3.4B). The difference in control values in all forms of Cu is due to different samples being taken from different mussel tissues (different experiments). A statistical comparison between the treatment groups of each Cu form and the respective control indicated that there was no significant difference in Cu concentrations in the treatment groups, compared to the respective controls. Furthermore, another statistical comparison between NPs results form compared to other forms results (MPs and salt) showed that there was no significant difference in Cu concentrations in the released form NPs in the exposure medium (seawater) compared to MPs and Cu salt (one-way ANOVA, $P<0.001$, followed by Tukey test) (Figure 3.4A). However, there is a significant difference in Cu concentrations in *M. edulis* exposed to CuO NPs, when compared to the other forms of Cu (salt $CuSO_4$) (Figure 3.4B).

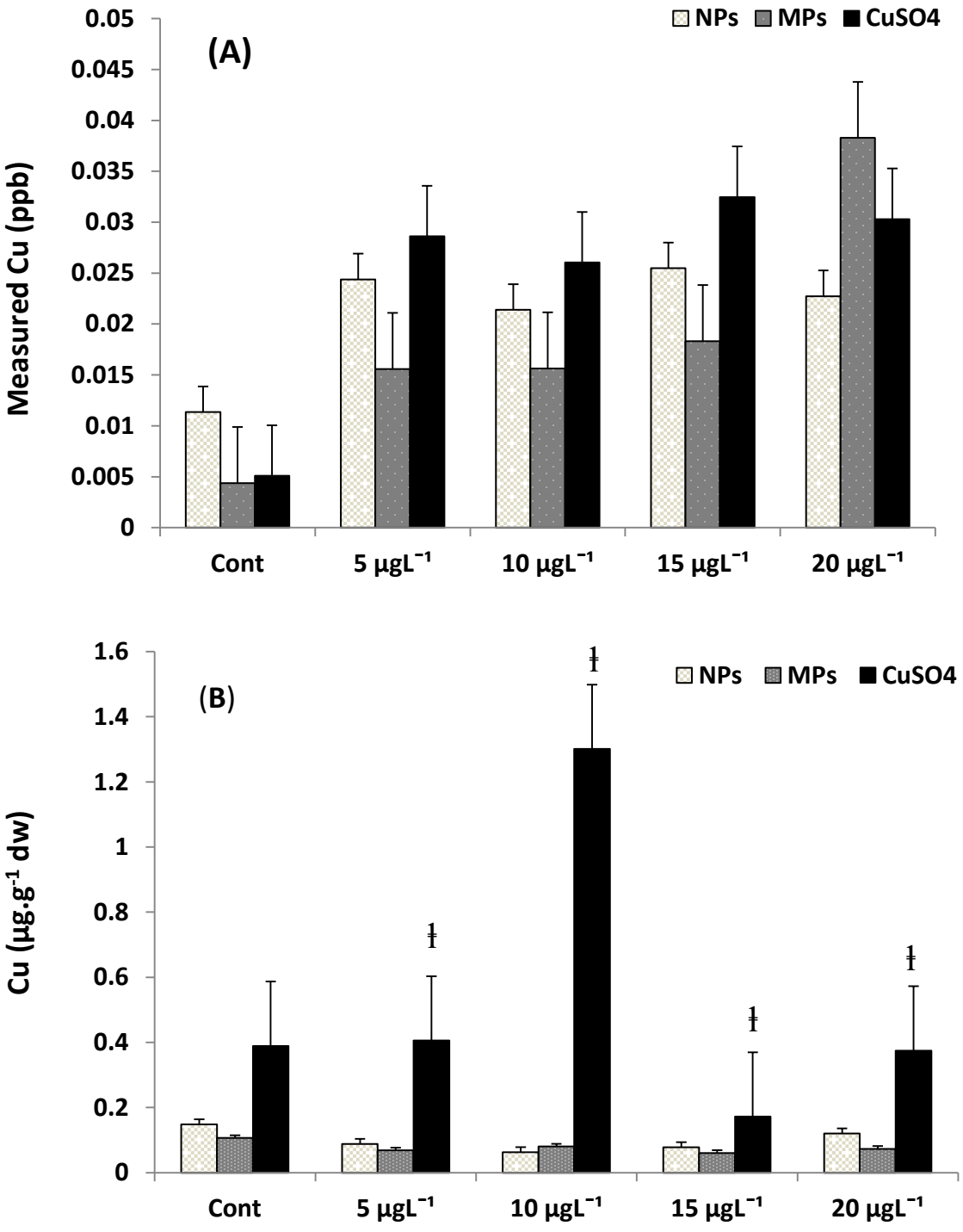


Figure 3.4: Cu concentrations measured by inductively coupled plasma optical emission spectrometry (ICPOES) after being exposed individually to CuO (NPs and MPs) and the salt form ($CuSO_4$). (A) Cu remained in exposure media (seawater). (B) Cu uptake by *M. edulis* mussels. (ǂ) A statistically significant difference between Cu salt compared to the nano form ($p<0.001$, means ± standard error, n=5).

3.2.2 Cell viability

Both cell viability results that were obtained from flow cytometry (FC) and trypan blue techniques showed that cell viability decreased gradually in the haemolymph cells of *M. edilus* when exposed separately to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations 5, 10, 15 and 20µgL^{-1} for 72 hours (Figure 3.5, Table 3.2 and Table 3.3). Flow cytometry (FC) expressed the population of live and dead cells as peaks, which reflect the viability of the mussel haemocytes, while the trypan blue approach only expressed the percentage of live and dead cells under the normal microscope and dead cells appeared in blue colour by taking up the blue dye. Statistically, there was no significant difference between results that obtained by either technique (flow cytometry (FC) and trypan blue) for all experiments.

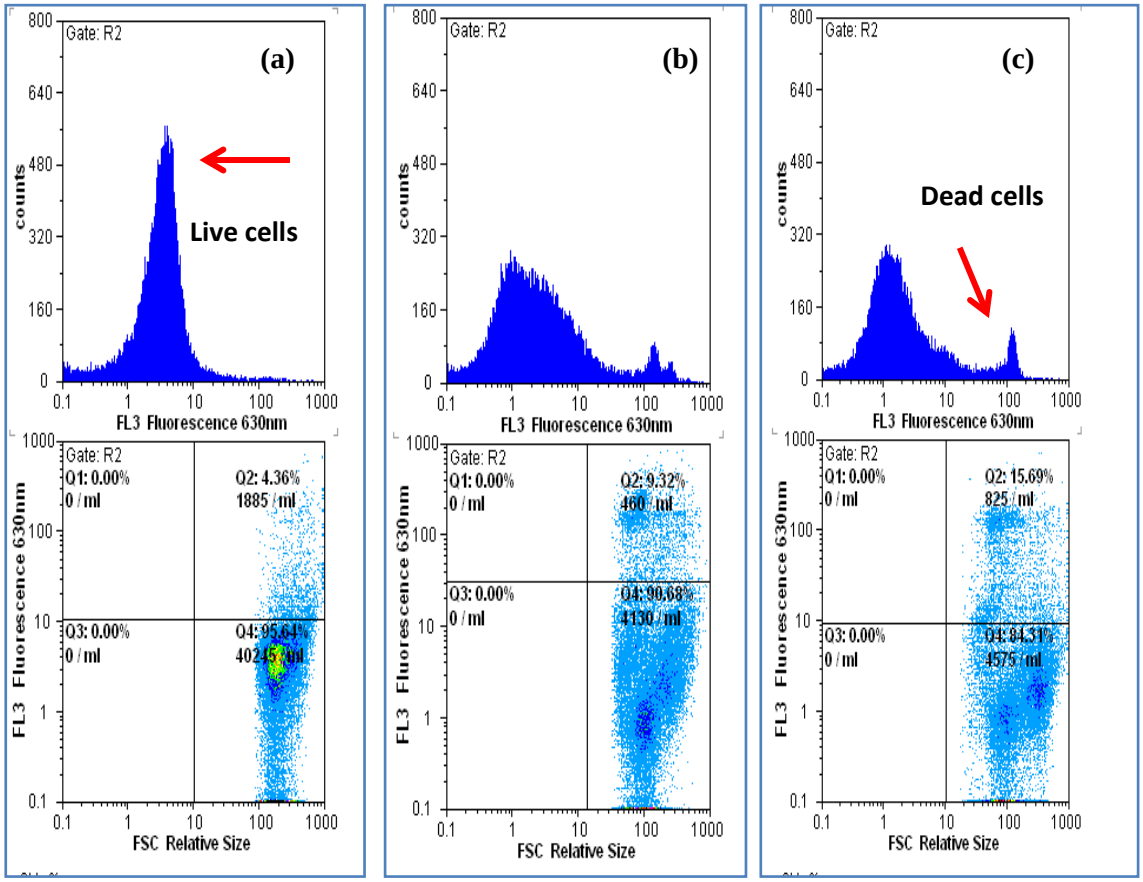


Figure 3.5: An example of the flow cytometry measurement, which showed the percentage of live haemolymph cells of the mussels exposed to different concentrations of CuO NPs. The highest peak expresses live cells, while the lowest peak expresses dead cells (illustrated by Red arrows) (n=2). (a) Control. (b) 10µgL^{-1}. (c) 20µgL^{-1}.

Table 3.2: The cell viability results for haemolymph cells of *M. edilus* exposed to different concentrations of different forms of Cu, measured by Flow cytometry (FC) (n=2).

Cu Concentration	Flow cytometry measurements		
	CuO NPs	CuO MPs	$CuSO_4$
Control	95.00%	96.16%	94.05%
5µgL^{-1}	89.75%	91.35%	93.31%
10µgL^{-1}	82.36%	87.35%	90.22%
15µgL^{-1}	78.39%	87.15%	84.82%
20µgL^{-1}	75.97%	84.52%	85.61%

Table 3.3: The cell viability results for haemolymph cells of *M. edilus* exposed to different concentrations of different forms of Cu, measured by Trypan blue (n=2).

Cu Concentration	Trypan blue measurements		
	CuO NPs	CuO MPs	$CuSO_4$
Control	96%	95%	96%
5µgL^{-1}	87%	89%	91%
10µgL^{-1}	80%	83%	89%
15µgL^{-1}	75%	79%	85%
20µgL^{-1}	73%	78%	82%

3.2.3 DNA damage

Comet assay results showed that there is a significant increase in DNA damage in both haemocytes and gill cells of *M. edilus* following exposure to 5, 10, 15 and 20µgL^{-1} as nominal concentrations of CuO NPs, CuO MPs and $CuSO_4$ for 72 hours; no dead animals were observed during the experiments (Figure 3.6 and Figure 3.7 A and B). A statistical comparison between the treatment groups of both types of cells (haemolymph and gill cells) and the control indicated that there was a significant increase in DNA damage in both types of cells in the treatment groups, compared to the respective controls, and also between each treatment group in a concentration response manner. Furthermore gill cells received more DNA damage than haemolymph cells in *M. edilus* mussels (one-way ANOVA, P<0.001, followed by Tukey test). The DNA damage was significantly higher in the cells of *M. edilus* following exposure CuO NPs than the cells exposed to CuO MPs and $CuSO_4$ at all selected concentrations (apart from 5µgL^{-1}) and these results were consistent with cell viability results (Figure 3.6 and Figure 3.7 A and B).

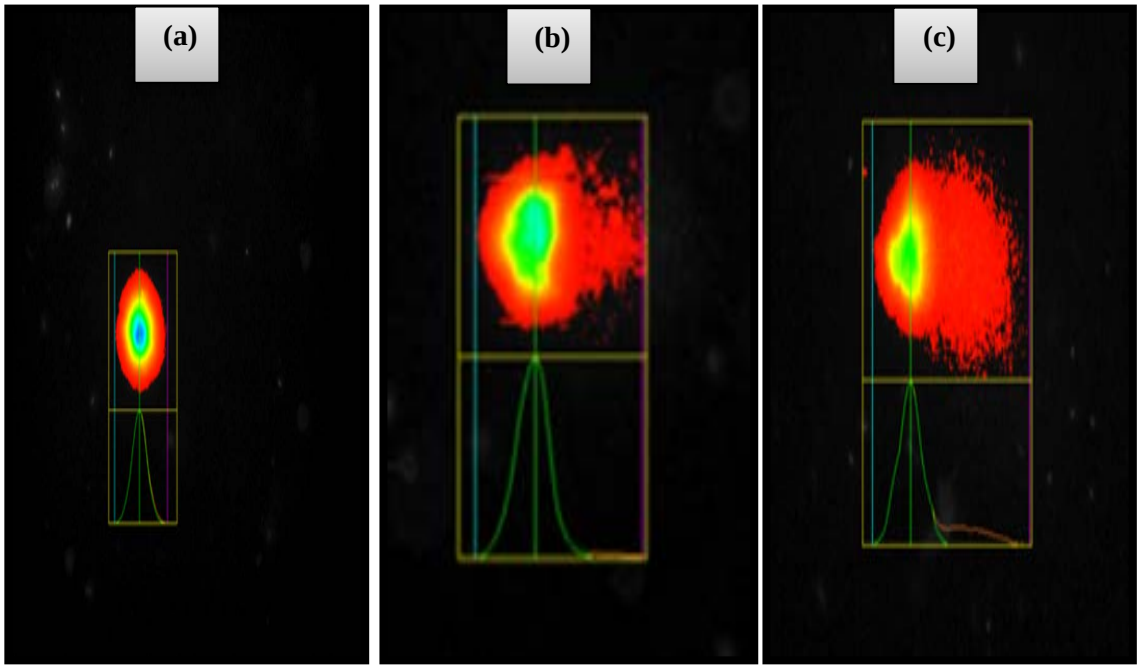


Figure 3.6: Images of the DNA damage in the DNA tail of mussel cells caused by different concentrations of CuO NPs, using Zeiss Axiophot microscope (the magnification was 40x/0.75) .(a) Control. (b) 10µgL^{-1}. (c) 20µgL^{-1}.

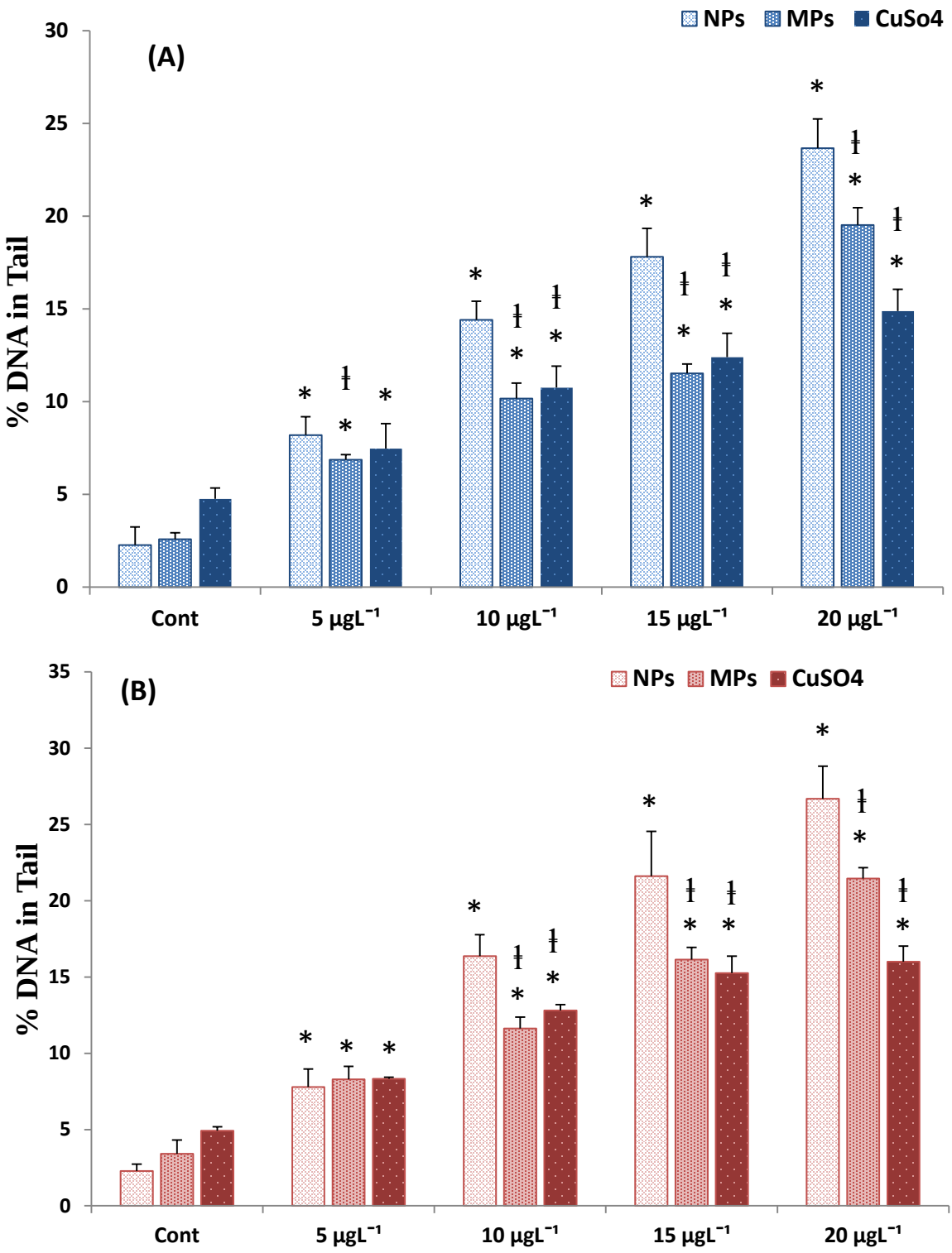


Figure 3.7: DNA damage in *M. edulis* mussels exposed to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations. (A) Haemolymph cells. (B) Gill cells. (*) A statistically significant difference compared to the respective control in each particle. (ǂ) A statistically significant difference between micro or Cu salt compared to the nano form ($p<0.001$, means ± standard deviation, n=5).

3.2.4 Oxidative stress

Oxidative stress was measured by assessing the superoxide dismutase activity (SOD) and lipid peroxidation in the gill of *M. edulis* using SOD assay and TBARS assay, respectively. The results showed that there was a concentration-dependent increase in SOD activity (expressed as percentage inhibition) and lipid peroxidation (expressed as TBARS nMol mg protein^{-1}) and therefore oxidative stress in gill cells of *M. edilus* in vivo exposed to 5, 10, 15 and 20µgL^{-1} nominal concentrations of CuO NPs, CuO MPs and $CuSO_4$ for 72 hours (Figure 3.8 and Figure 3.9).

Statistically, there was a significant difference observed in SOD activity and lipid peroxidation in gill cells of *M. edulis* between the treatment groups and the respective controls, and also between each treatment group. In addition, a statistical comparison between the toxicity of CuO NPs compared to other forms MPs and $CuSO_4$ indicated that SOD activity and lipid peroxidation were significantly higher in gill cells exposed to CuO NPs (apart from 10µgL^{-1}) compared to those exposed to other forms of Cu (MPs and $CuSO_4$), which were consistent with cell viability and DNA damage results (Figure 3.8 and Figure 3.9).

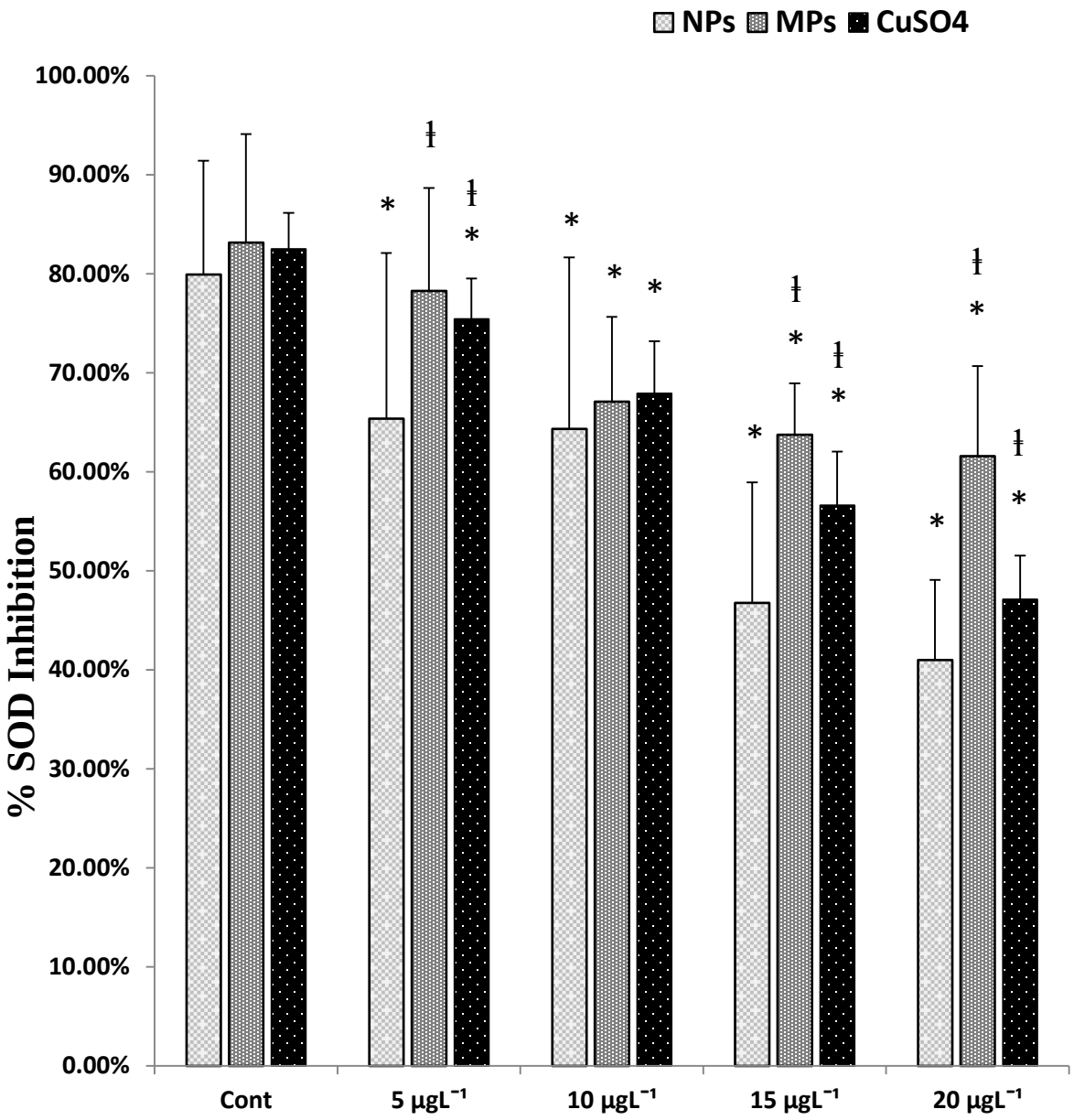


Figure 3.8: Activity of superoxide dismutase (SOD) (expressed as percentage of inhibition) in *M. edulis* gill cells exposed to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations. (*) A statistically significant difference compared to the control group in each particle. (ǂ) A statistically significant difference between micro or Cu salt compared to nano form ($p<0.001$, means ± standard deviation, n=5).

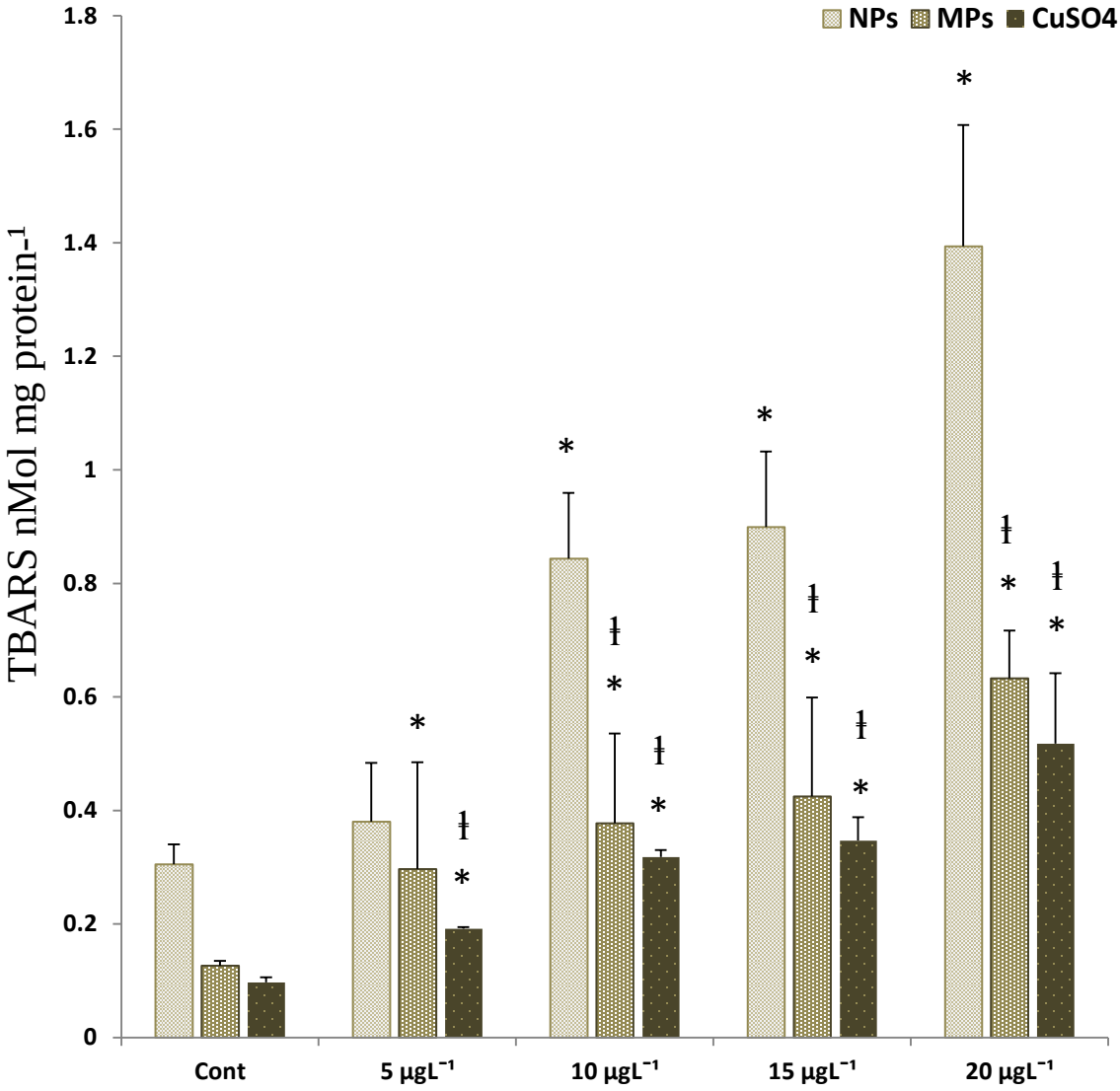


Figure 3.9: The level of thiobarbituric acid reactive substances (expressed as TBARS nMol mg protein^{-1}) in gill cells of *M. edulis* exposed to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations. The difference in control values is due to different samples taken from different mussel gills (different experiments). (*) A statistically significant difference compared to the control group in each particle. (ǂ) A statistically significant difference between micro or Cu salt compared to nano form ($p<0.001$, means ± standard deviation, n=5).

3.3 *M. modiolus* mussels

3.3.1 Cu concentrations

The total Cu concentration in both exposure medium (seawater) and *M. modiolus* tissues were analysed after separate exposure to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at the nominal concentrations (5, 10, 15 and 20µgL^{-1}) for 72 hours, using inductively coupled plasma optical emission spectrometry (ICPOES) (Figure 2.14). Results indicated that a consistent higher total amount of copper was measured in the particulate exposures when compared to the salt treatment (Figure 3.10A). In contrast, the copper uptake by *M. modiolus* in the different copper exposures, was higher in the copper salt exposure at lower concentrations but higher for the NP treatment at the highest exposure concentration (20µgL^{-1}) (Figure 3.10B). The difference in control values in all forms of Cu is due to different samples taken from different mussel tissues (different experiments). A statistical comparison between the treatment groups of each Cu form and the respective control indicated that there was no significant difference in Cu concentrations in the treatment groups, compared to the respective controls. Furthermore, another statistical comparison between NPs results form compared to other forms results (MPs and salt) showed that there now no significant difference in Cu concentrations released from NPs either in the exposure medium (seawater) and in the mussel tissues compared to MPs and Cu salt (one-way ANOVA, $P<0.001$, followed by Tukey test) (Figure 3.10 A & B).

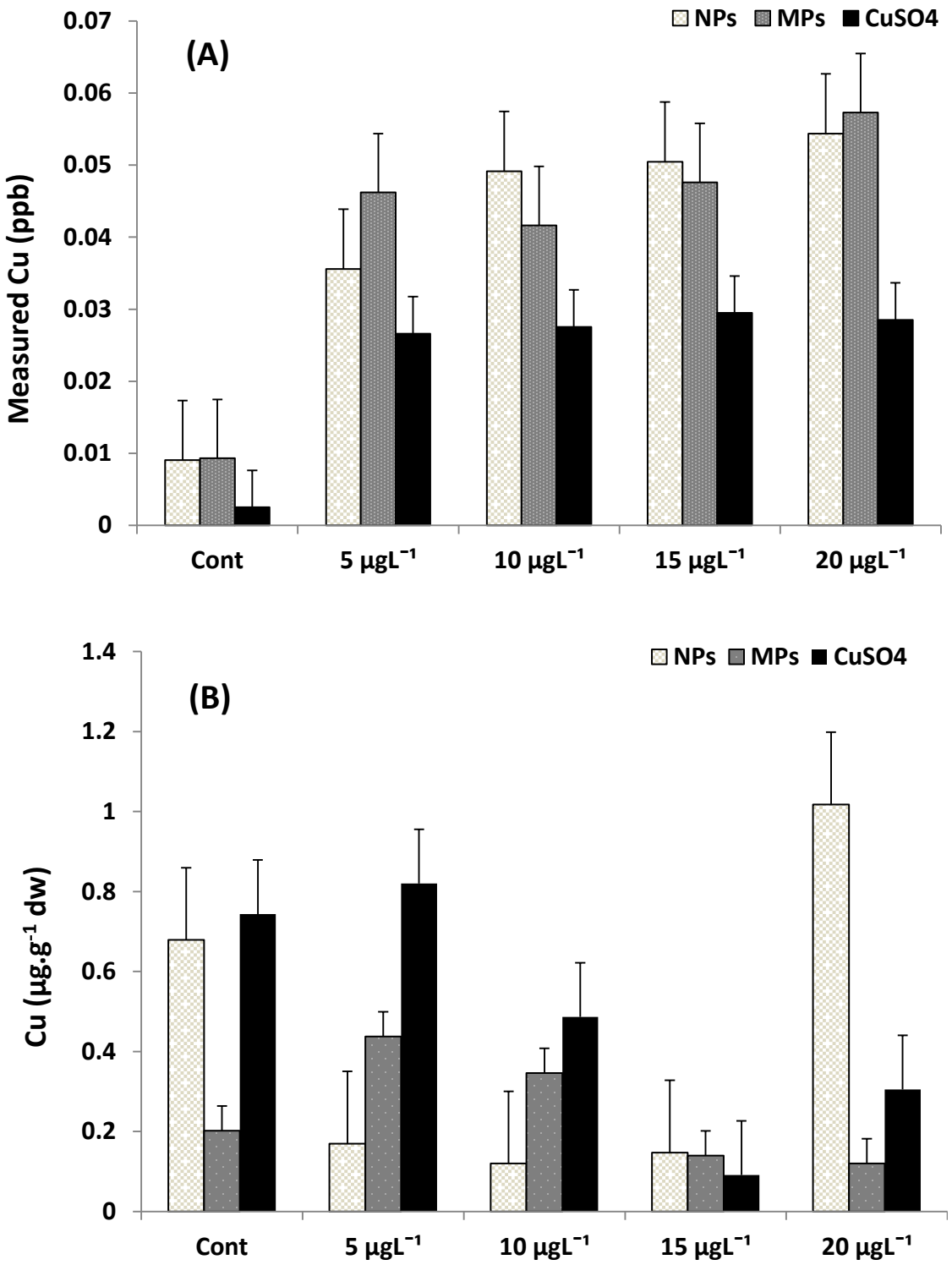


Figure 3.10: Cu concentrations measured by inductively coupled plasma optical emission spectrometry (ICPOES) after mussel exposures to CuO (NPs and MPs) and the salt form ($CuSO_4$) (A) Cu in exposure medium (seawater). (B) Cu uptake by *M. modiolus* mussels. (Means ±standard error, n=5).

3.3.2 Cell viability

Both cell viability results that were obtained from flow cytometry (FC) and trypan blue techniques showed that cell viability decreased gradually in the haemolymph cells of *M. modiolus* when exposed separately to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations (5, 10, 15 and 20$\mu gL^{-1)}$ for 72 hours (Figure 3.5, Table 3.4 and Table 3.5). Statistically, there was no significant difference between results obtained by either technique (flow cytometry and trypan blue) for all experiments.

Table 3.4: The cell viability results for haemolymph cells of *M. modiolus* exposed to different concentrations of different forms of Cu, measured by Flow cytometry (n=2).

Cu Concentration	**Flow cytometry measurements**		
	CuO NPs	**CuO MPs**	**$CuSO_4$**
Control	**97.08%**	**96.29%**	**91.87%**
5μgL^{-1}	**95.03%**	**92.45%**	**90.14%**
10μgL^{-1}	**86.07%**	**88.44%**	**85.24%**
15μgL^{-1}	**80.22%**	**89.14%**	**84.25%**
20μgL^{-1}	**79.23%**	**87.81%**	**84.19%**

Table 3.5: The cell viability results for haemolymph cells of *M. modiolus* exposed to different concentrations of different forms of Cu, measured by Trypan blue (n=2).

Cu Concentration	Trypan blue measurements		
	CuO NPs	**CuO MPs**	**$CuSO_4$**
Control	**94%**	**96%**	**94%**
5µgL^{-1}	**90%**	**91%**	**89%**
10µgL^{-1}	**87%**	**89%**	**86%**
15µgL^{-1}	**82%**	**85%**	**84%**
20µgL^{-1}	**79%**	**82%**	**82%**

3.3.3 DNA damage

Comet assay results showed that there was a significant increase in DNA damage in both haemocytes and gill cells of *M. modiolus* following a 72 hours exposure to 5, 10, 15 and 20µgL^{-1} nominal concentrations of CuO NPs, CuO MPs and $CuSO_4$ and no dead animals were observed during the experiments (Figure 3.6 and Figure 3.11 A and B). A statistical comparison between the treatment groups of both types of cells (haemolymph and gill cells) and the control indicated that there was a significant increase in DNA damage in both types of cells in the treatment groups, compared to the respective controls, and also between each treatment group in a concentration response manner. Furthermore gill cells received more DNA damage than haemolymph cells in *M. modiolus* mussels (one-way ANOVA, $P<0.001$, followed by Tukey test). The DNA damage was higher in the cells of *M. modiolus* following exposure to CuO NPs than the cells exposed to CuO MPs and $CuSO_4$ at all selected concentrations (apart from 5µgL^{-1}) (Figure 3.11 A and B).

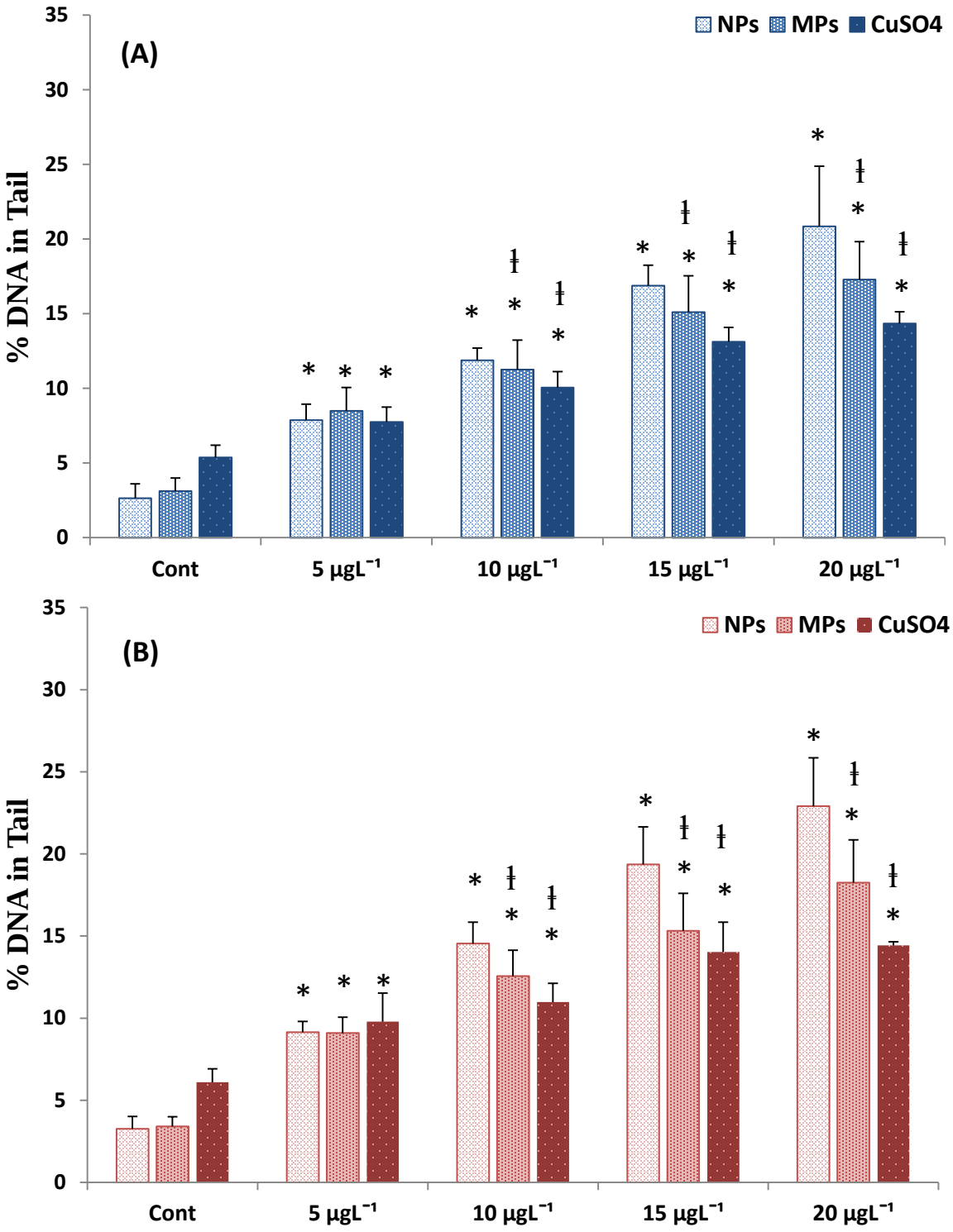


Figure 3.11: DNA damage in *M. modiolus* mussels exposed to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations. (A) Haemolymph cells. (B) Gill cells. (*) A statistically significant difference compared to the respective control in each particle. (ǂ) A statistically significant difference between micro or Cu salt compared to nano form ($p<0.001$, means ± standard deviation, n=5)

3.3.4 Oxidative stress

Similarly, oxidative stress was measured by assessing the superoxide dismutase activity (SOD) and lipid peroxidation in the gill of *M. modiolus* using SOD assay and TBARS assay, respectively. The results showed that there was a concentration-dependent increase in SOD activity (expressed as percentage inhibition) and Lipid peroxidation (expressed as TBARS nMol mg protein^{-1}) and therefore oxidative stress in gill cells of *M. modiolus* in vivo exposed to 5, 10, 15 and 20µgL^{-1} nominal concentrations of CuO NPs, CuO MPs and $CuSO_4$ for 72 hours (Figure 3.12 and Figure 3.13). Statistically, there was a significant difference observed in SOD activity and lipid peroxidation in gill cells of *M. modiolus* between the treatment groups and the respective controls, and also between each treatment group. In addition, a statistical comparison between the toxicity of CuO NPs compared to other forms MPs and $CuSO_4$ indicated that no significant difference in SOD activity in gill cells exposed to all forms of Cu. While, lipid peroxidation was significantly higher in gill cells exposed to CuO NPs (apart from 5µgL^{-1}) compared to those exposed to other forms of Cu (MPs and $CuSO_4$) (Figure 3.12 and Figure 3.13).

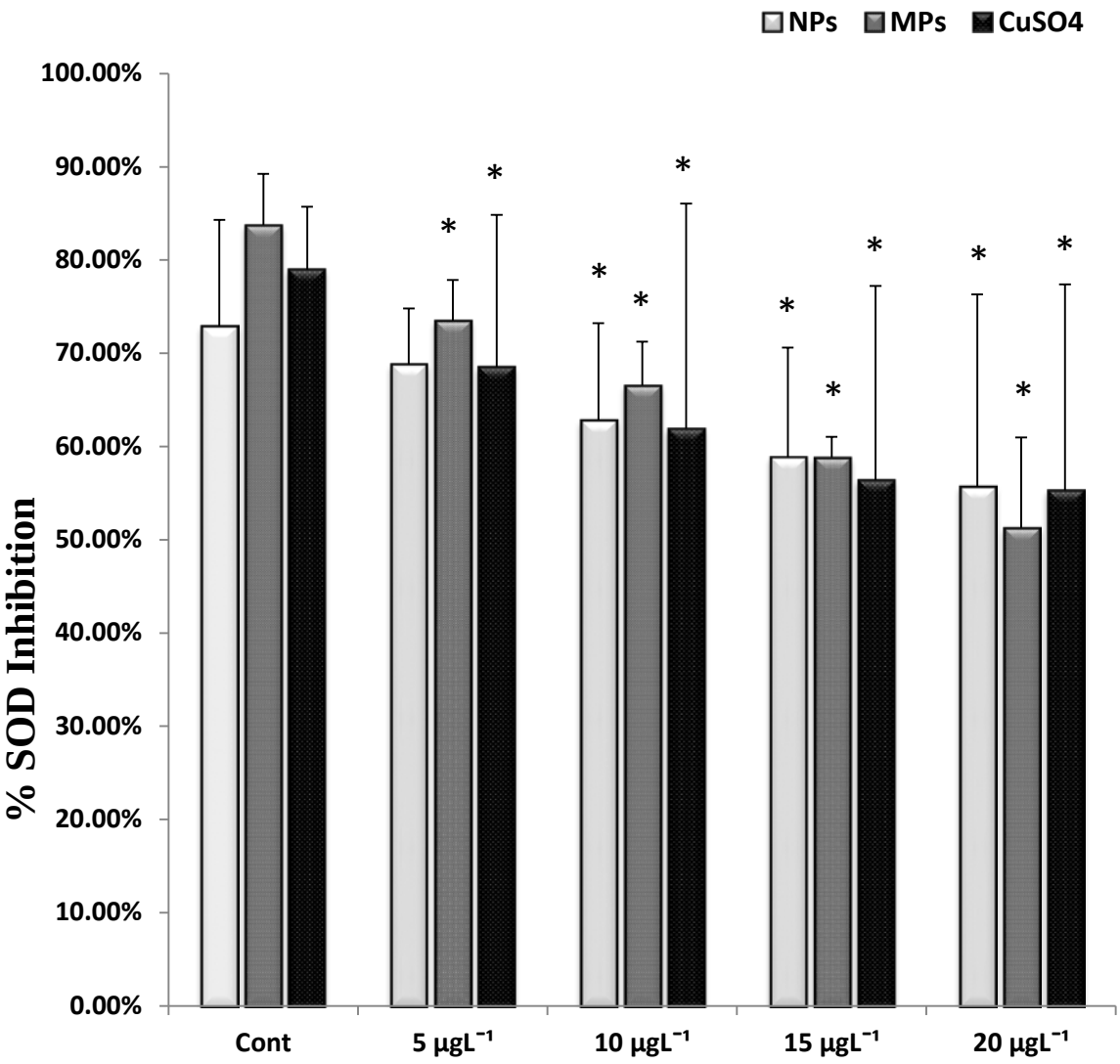


Figure 3.12: Activity of superoxide dismutase (SOD) (expressed as percentage of inhibition) in *M. modiolus* gill cells exposed to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations. (*) A statistical significant difference compared to the control group in each particle ($p<0.001$, means ± standard deviation, n=5).

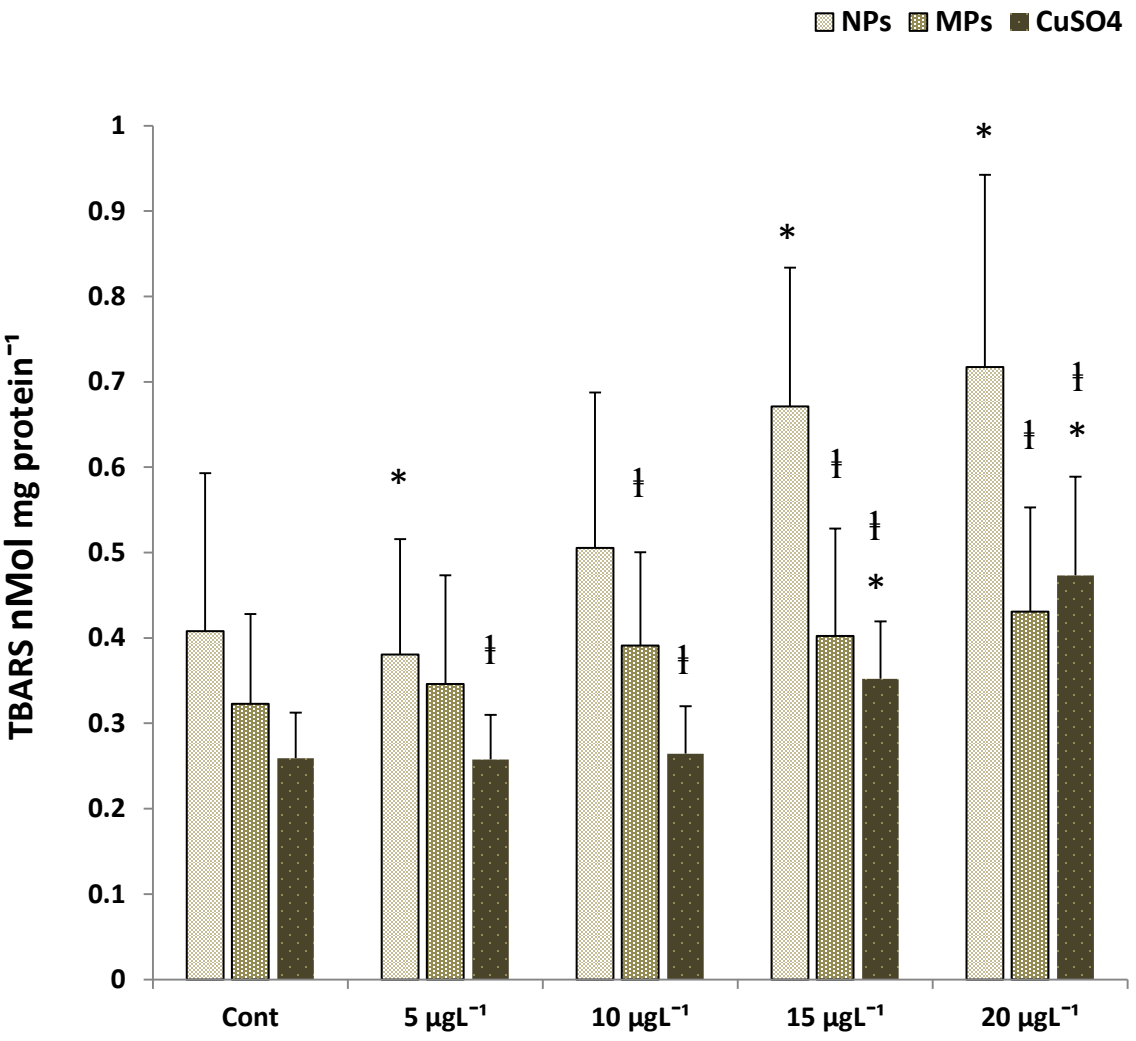


Figure 3.13: The level of thiobarbituric acid reactive substances (expressed as TBARS nMol mg protein^{-1}) in gill cells of *M. modiolus* exposed to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations. (*) A statistically significant difference compared to the control group in each particle. (ɬ) A statistically significant difference between micro or Cu salt compare to nano form ($p<0.001$, means ± standard deviation, n=5).

3.4 Comparison between *M. edulis* and *M. modiolus*

3.4.1 Cell viability

The cell viability results obtained from flow cytometry and trypan blue techniques illustrated that cell viability of haemolymph cells of both mussels (*M. edilus* and *M. modiolus*) decreased gradually after exposed to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) separately at nominal concentrations (5, 10, 15 and 20µgL$^{-1)}$ for 72 hours (Table 3.6 (A, B & C)). There was a significant difference in the cell viability in both mussels exposed to all forms of Cu. Furthermore, there was no significant difference between results obtained by either technique (flow cytometry and trypan blue) for all experiments (Table 3.6 (A, B & C)).

Table 3.6: The cell viability results for haemolymph cells of both mussel species (*M. edilus* and *M. modiolus)* exposed to different concentrations of different forms of Cu (n=2). (A) CuO NPs. (B) CuO MPs and (C) $CuSO_4$.

(A)	*M. edulis*		*M. modiolus*	
Concentration CuO NPs	**Flow cytometry**	**Trypan blue**	**Flow cytometry**	**Trypan blue**
Control	**95.00%**	**96%**	**97.08%**	**94%**
5µgL^{-1}	**89.75%**	**87%**	**95.03%**	**90%**
10µgL^{-1}	**82.36%**	**80%**	**86.07%**	**87%**
15µgL^{-1}	**78.39%**	**75%**	**80.22%**	**82%**
20µgL^{-1}	**75.97%**	**73%**	**79.23%**	**79%**

(B) Concentration CuO MPs	*M. edulis*		*M. modiolus*	
	Flow cytometry	Trypan blue	Flow cytometry	Trypan blue
Control	96.16%	95%	96.29%	96%
5µgL^{-1}	91.35%	89%	92.45%	91%
10µgL^{-1}	87.35%	83%	88.44%	89%
15µgL^{-1}	87.15%	79%	89.14%	85%
20µgL^{-1}	84.52%	78%	87.81%	82%

(C) Concentration $CuSO_4$	*M. edulis*		*M. modiolus*	
	Flow cytometry	Trypan blue	Flow cytometry	Trypan blue
Control	94.05%	96%	91.87%	94%
5µgL^{-1}	93.31%	91%	90.14%	89%
10µgL^{-1}	90.22%	89%	85.24%	86%
15µgL^{-1}	84.82%	85%	84.25%	84%
20µgL^{-1}	85.61%	82%	84.19%	82%

3.4.2 DNA damage

A significant increase in DNA damage was observed in both haemocytes and gill cells of both mussels *M. edulis* and *M. modiolus* following exposure to 5, 10, 15 and 20µgL^{-1} nominal concentrations of CuO NPs, CuO MPs and $CuSO_4$ for 72 hours (Figure 3.14–Figure 3.16). Gill cells were more sensitive than haemolymph cells in both mussels receiving higher DNA damage (one-way ANOVA, $P<0.001$, followed by Tukey test). Furthermore, a statistical comparison was carried out between the levels of DNA damage in cells of both mussels after exposure to each form of Cu. The DNA damage was higher in *M. edulis* cells exposed to CuO NPs and $CuSO_4$ than in *M. modiolus* cells (Figure 3.14 and Figure 3.16), while the *M. modiolus* cells received higher DNA damage after exposure to CuO MPs than *M. edulis* cells (apart from 20µgL^{-1}) (Figure 3.15).

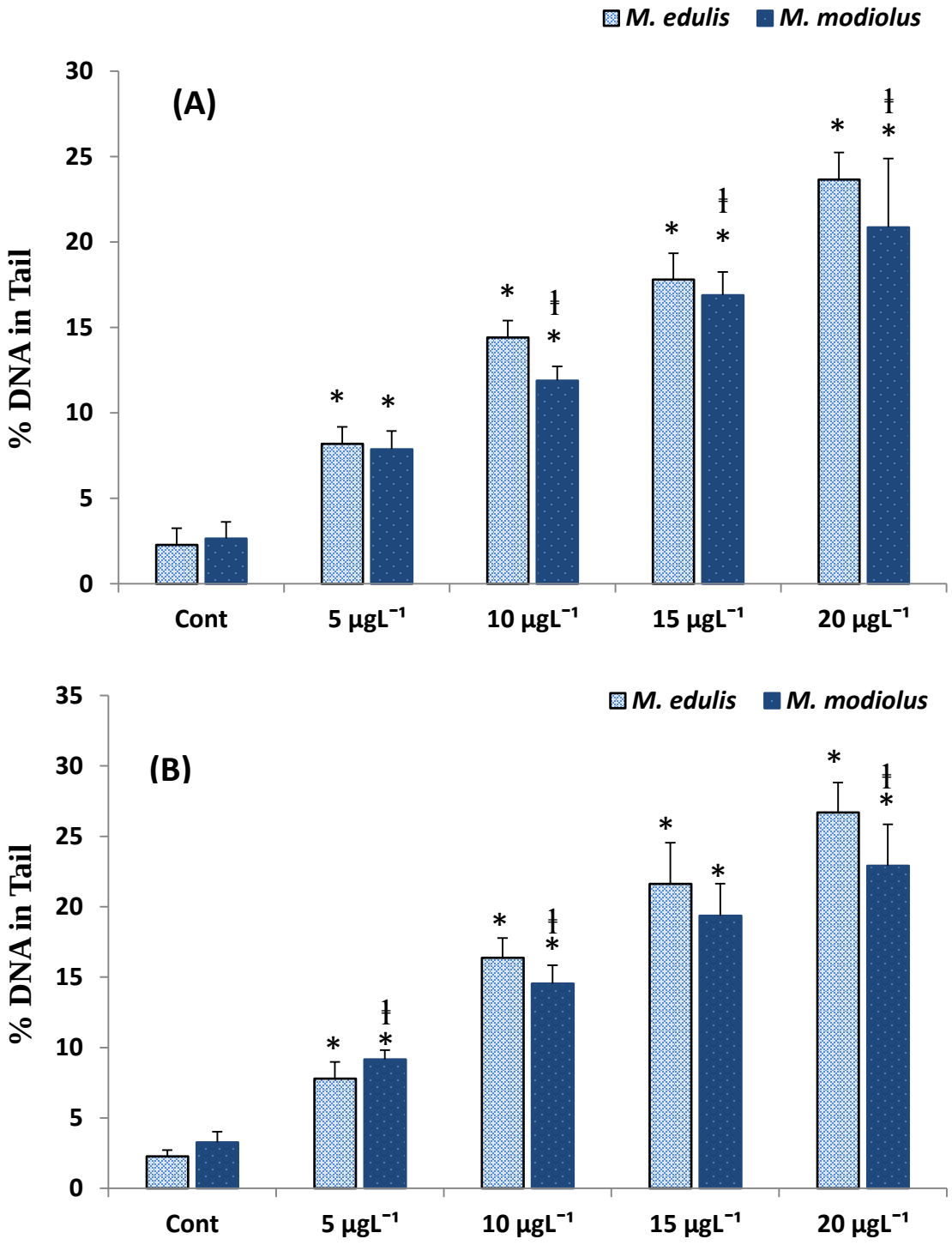


Figure 3.14: DNA damage in both mussel species *(M. edulis* and *M. modiolus*) exposed to CuO NPs at nominal concentrations. (A) Haemolymph cells. (B) Gill cells. (*) A statistically significant difference compared to the respective control in each mussel. (ǂ) A statistically significant difference between mussel species in each concentration ($p<0.001$, means ± standard deviation, n=5).

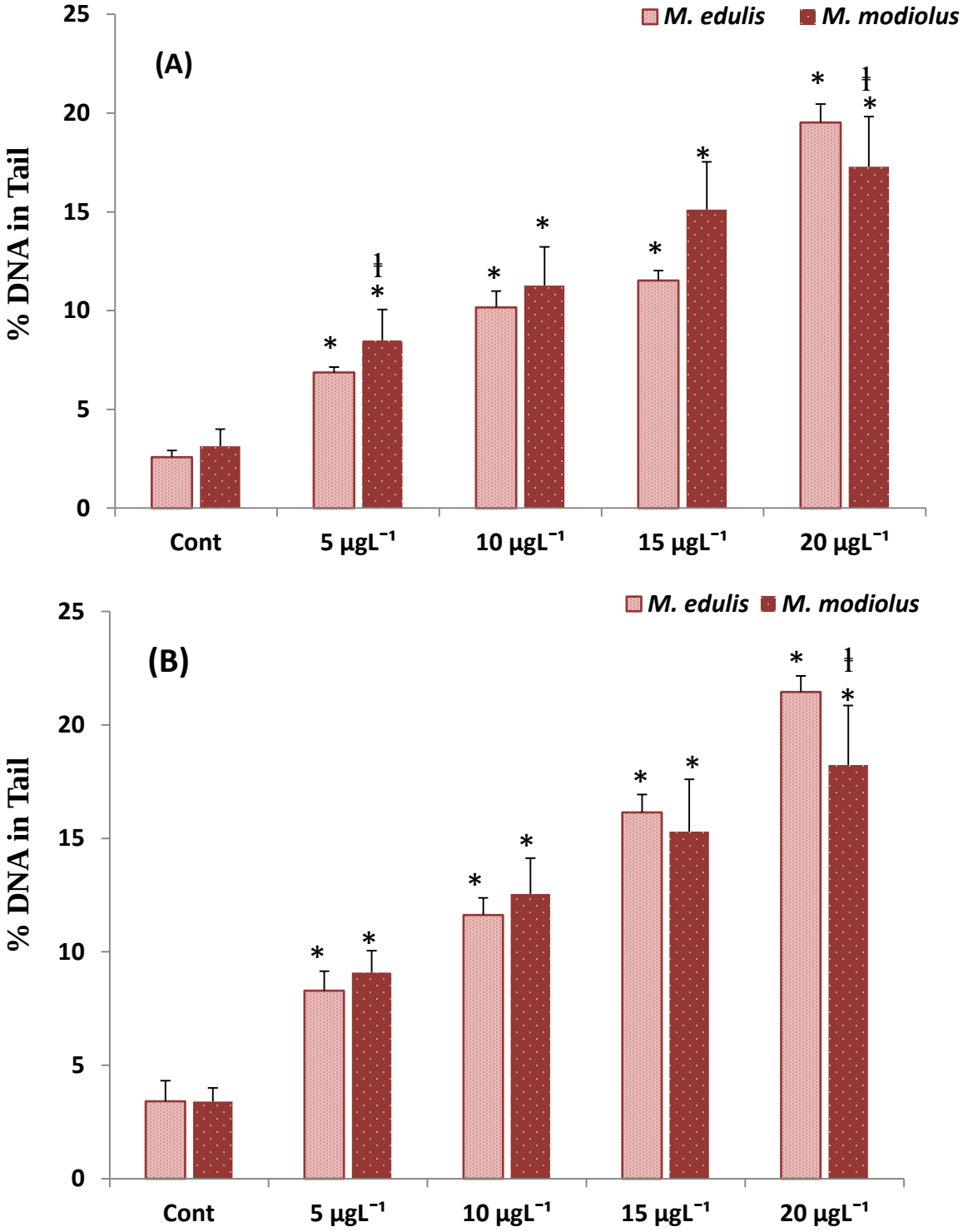


Figure 3.15: DNA damage in both mussel species (*M. edulis* and *M. modiolus*) exposed to CuO MPs at nominal concentrations. (A) Haemolymph cells. (B) Gill cells. (*) A statistically significant difference compared to the respective control in each mussel. (ǂ) A statistically significant difference between mussel species in each concentration ($p<0.001$, means ± standard deviation, n=5).

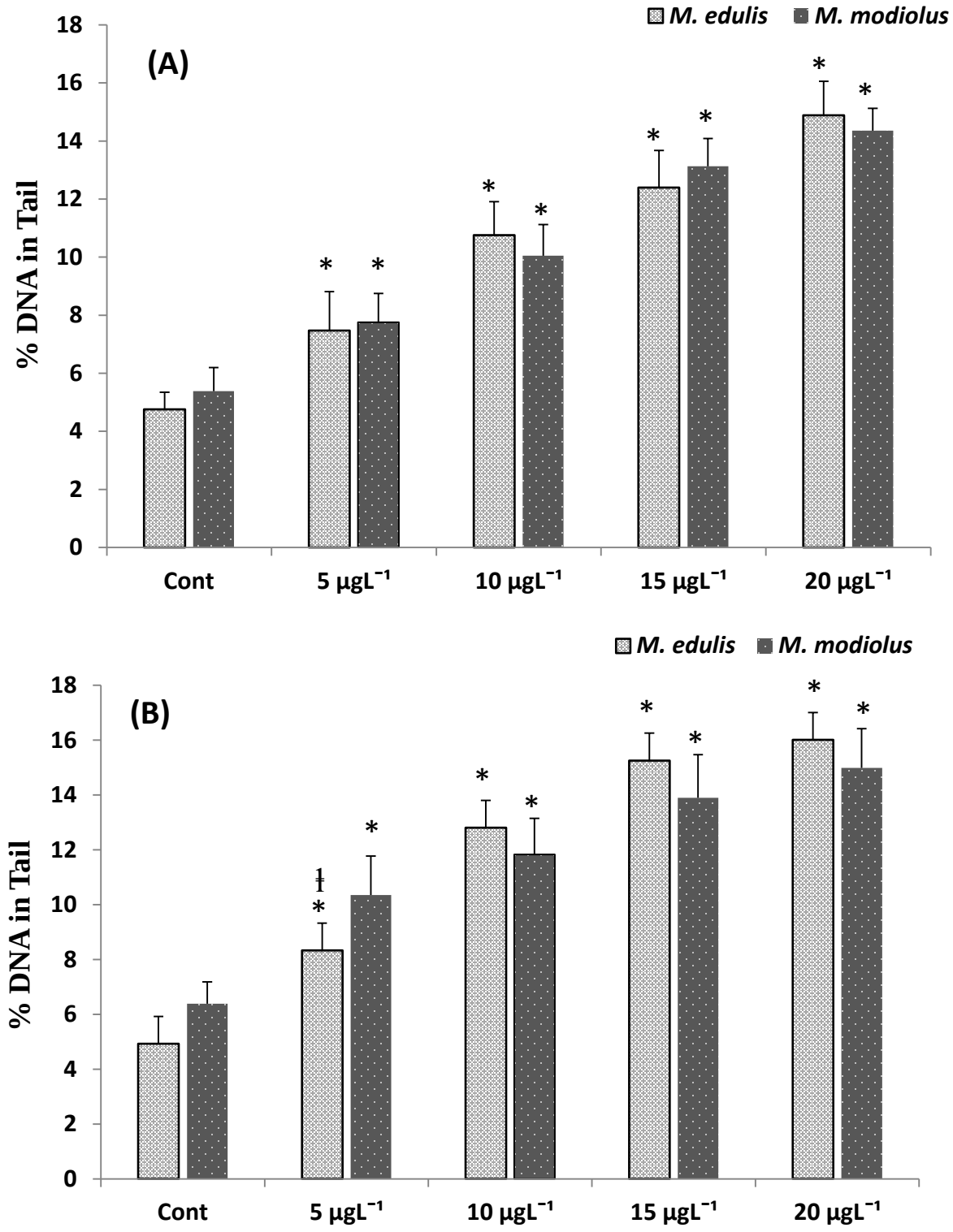
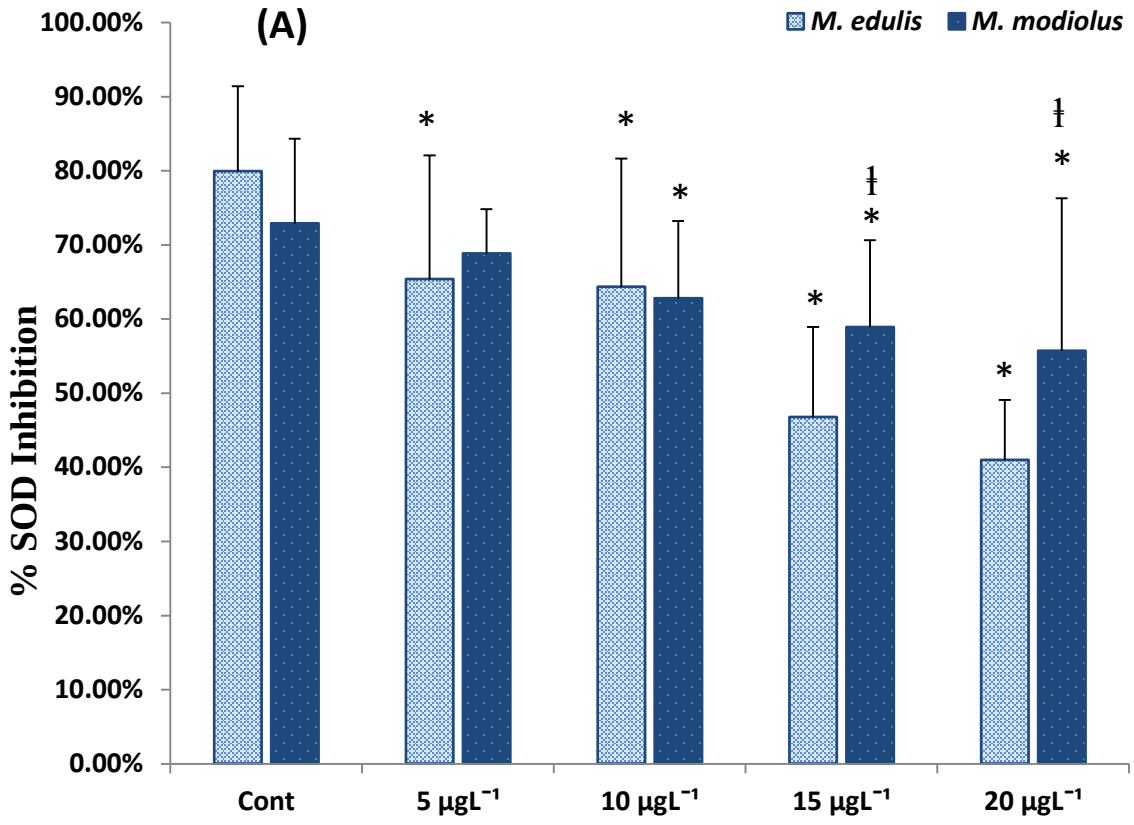


Figure 3.16: DNA damage in both mussel species (*M. edulis* and *M. modiolus*) exposed to $CuSO_4$ at nominal concentrations. (A) Haemolymph cells. (B) Gill cells. (*) A statistically significant difference compared to the respective control in each mussel. (ǂ) A statistically significant difference between mussel species in each concentration form ($p<0.001$, means ± standard deviation, n=5).

3.4.3 Oxidative stress

The results of SOD and TBARS assays indicated that there was a concentration-dependent increase in SOD activity (expressed as percentage of inhibition) and Lipid peroxidation (expressed as TBARS nMol mg protein^{-1}) and therefore oxidative stress in gill cells of *M. edulis* and *M. modiolus* in vivo exposed to 5, 10, 15 and 20µgL^{-1} as nominal concentrations of CuO NPs, CuO MPs and $CuSO_4$ for 72 hours (Figure 3.17 and Figure 3.18). In addition, a statistical comparison was carried out between the activity of SOD and the level of lipid peroxidation in gill cells of both mussels after exposing to each forms of Cu. There was no significant difference in SOD activity and lipid peroxidation in both mussels exposed to all forms of Cu (Figure 3.17 and Figure 3.18). However, SOD activity and lipid peroxidation were significantly higher in *M. edulis* exposed to 15 and 20µgL^{-1} and 10, 20µgL^{-1} of CuO NPs respectively (Figure 3.17A and Figure 3.18A).



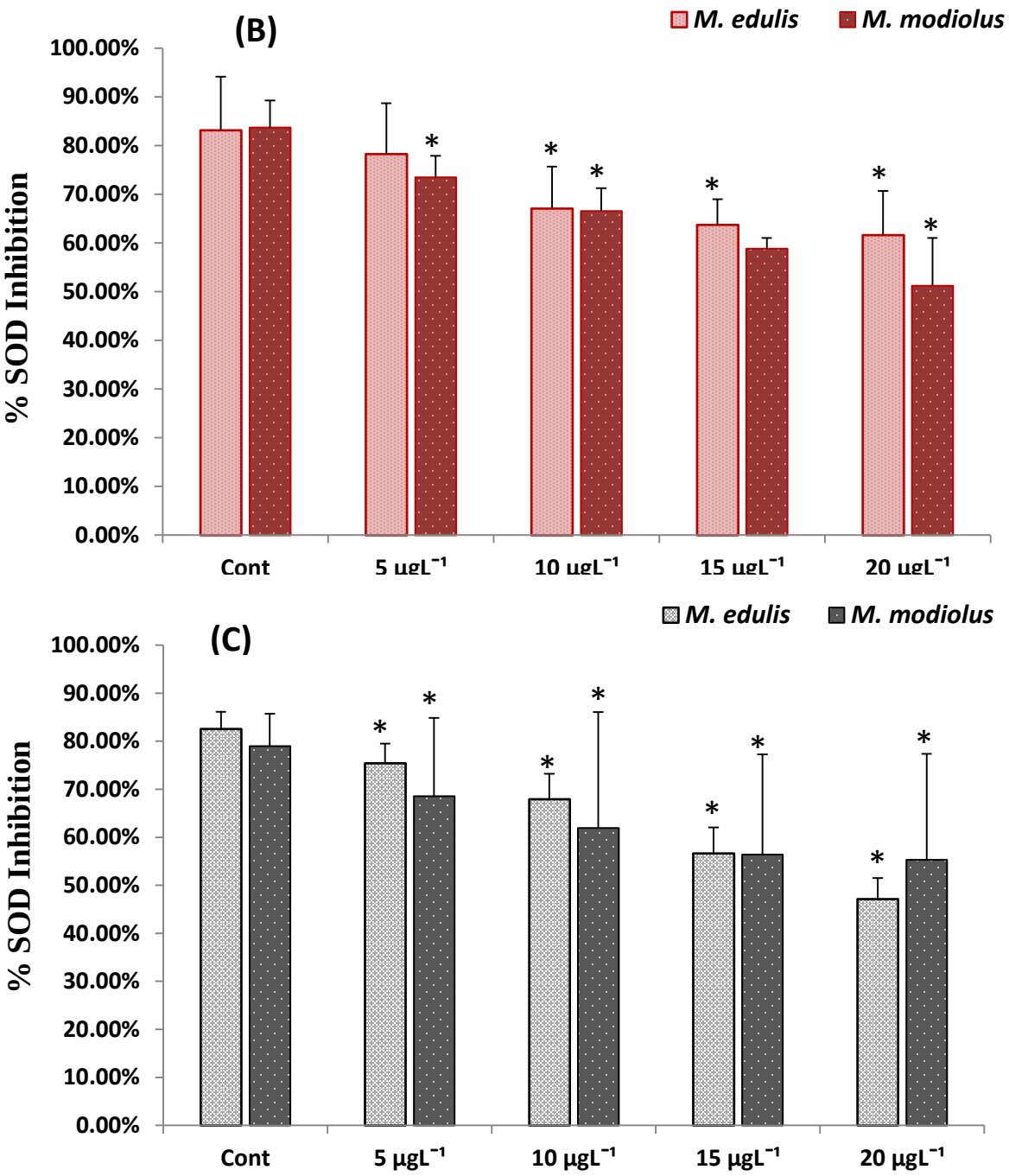
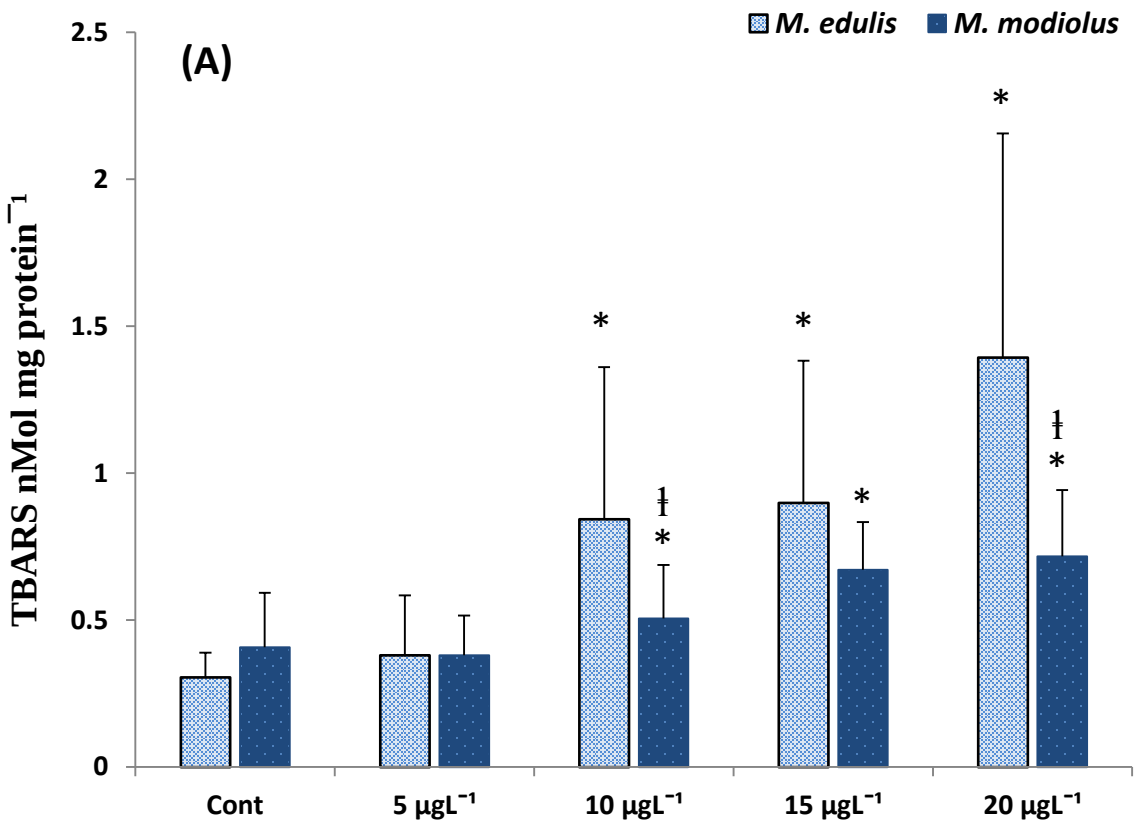
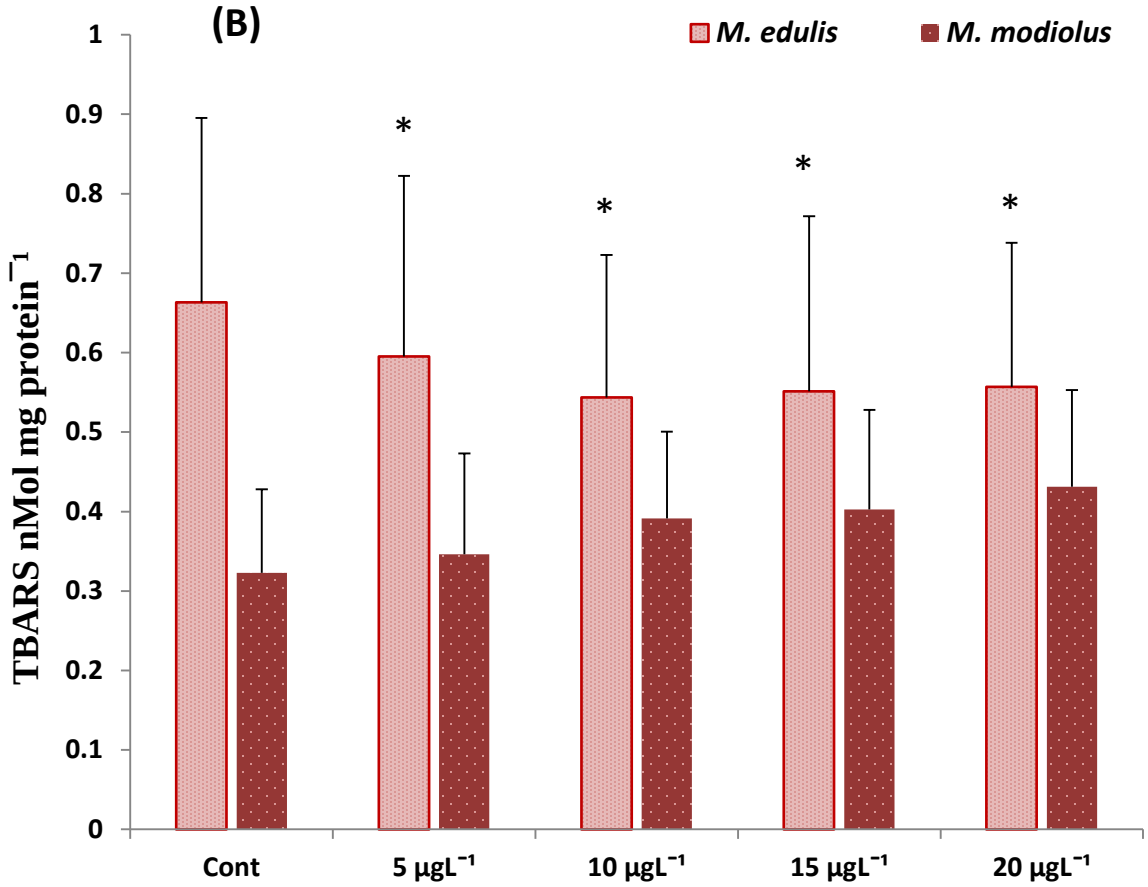


Figure 3.17: Activity of superoxide dismutase (SOD) (expressed as percentage of inhibition) in gill cells of both mussel species (*M. edulis* and *M. modiolus*) exposed to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations, (A) CuO NPs. (B) CuO MPs. (C) $CuSO_4$. (*) A statistically significant difference compared to the control group in each mussel. (ǂ) A statistically significant difference between mussel species in each concentration ((p<0.001, means ± standard deviation, n=5).


(A)
M. edulis
M. modiolus
TBARS nMol mg protein⁻¹
2.5
2
1.5
1
0.5
0
Cont
5 µgL⁻¹
10 µgL⁻¹
15 µgL⁻¹
20 µgL⁻¹


(B)
M. edulis
M. modiolus
TBARS nMol mg protein⁻¹
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cont
5 µgL⁻¹
10 µgL⁻¹
15 µgL⁻¹
20 µgL⁻¹

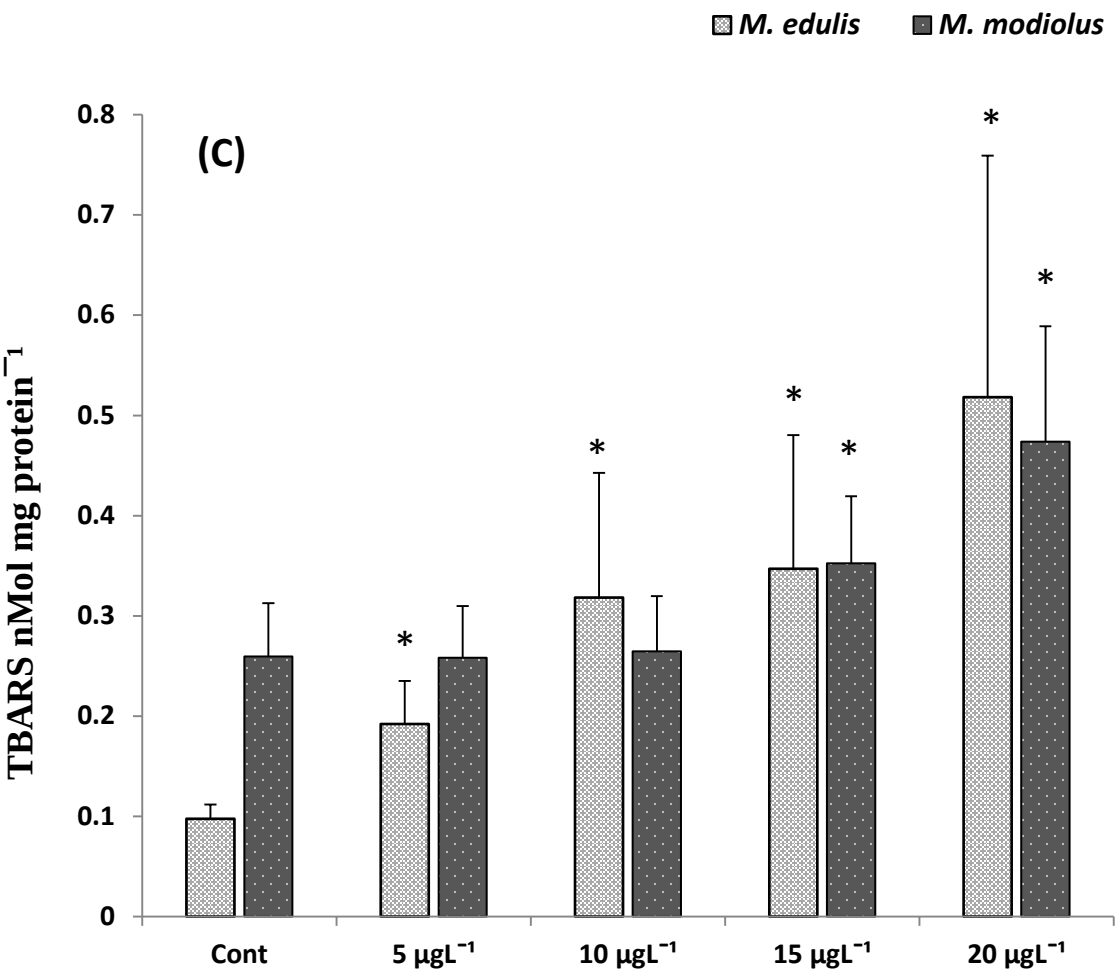


Figure 3.18: The level of thiobarbituric acid reactive substances (expressed as TBARS nMol mg protein^{-1}) in gill cells of both mussel species (*M. edulis* and *M. modiolus*) exposed to both forms of particulate CuO (NPs and MPs) and the salt form ($CuSO_4$) at nominal concentrations. (A) CuO NPs. (B) CuO MPs. (C) $CuSO_4$. (*) A statistically significant difference compared to the control group in each mussel. (ł) A statistically significant difference between mussel species in each concentration ($p<0.001$, means ± standard deviation, n=5).

4 DISCUSSION

4.1 Characterisation of CuO particles (NPs and MPs)

The ecotoxicity of engineered NPs can be affected by several innate physicochemical properties, such as charge, aggregation level, particle size, size distribution, and solubility, and environmental factors such as pH, salinity, and temperature (Buffet et al., 2011). Furthermore, phase purity, crystallinity, particle and cluster size, solubility, charge and surface chemistry are fundamental to express the homogeneity, stability, reactivity, biodurability, and potential fate of NPs in different media (Kahru et al., 2010). The size of the particles and their elemental composition are considered as the most important physicochemical properties of NPs, since they have a strong influence on the other properties such as surface area and aggregation level, as well as the behaviour of these particles in the media (Peralta-Videa et al., 2011). Addressing the characteristics of NPs is one of the main concerns when seeking to understand the toxicity of these particles (Siddiqui et al., 2013). Furthermore, the physical and chemical properties of metals, such as active surface charge, hardness, shape, chemical, and biological activity are also affected by changes in particle size (Ren et al., 2009).

Recently, various analytical methods and techniques have been used for characterising the physical and chemical features of NPs, such as microscopy, chromatography, spectroscopy, centrifugation, filtration and others (Farré et al., 2009). Examples of the these techniques including elemental analysis electron loss spectroscopy (EELS) and energy dispersive X-ray spectroscopy (EDS), selected area electron diffraction (SAED), near-field scanning optical microscope (NSOM), confocal laser scanning microscope (CLSM), scanning electron microscope (SEM) and atomic force microscope (AFM), transmission electron microscope (TEM) and dynamic light scattering (DLS). In the present study, the characteristics of CuO particulates (NPs and MPs) were identified using two common techniques: TEM and DLS, to determine the particles' shape, size, aggregation state and zeta potential. In the first test, both particle sizes of CuO (NPs and MPs) were prepared in seawater and observed under TEM (Figure 3.1 and Figure 3.2). This is a significant observation, because the deposition of salt crystals can obscure particles on the grids following evaporation. However, this process was not uniform

across the grid, and did allow observation of uninhibited particles, thereby allowing the characterisation of size and shape in the exposure medium.

4.1.1 TEM and DLS

Transmission electron microscope (TEM) is considered one of the most valuable tools given its high resolution, which allows the corroboration of structure, morphology, as well as diffraction data for NPs (Karlsson et al., 2009). The results which were obtained by TEM for the shape and size of CuO particles (NPs and MPs) showed that NPs were spherical in shape, with a size less than 50 nm, while MPs were seen as reticular shaped particles, with a size less than 5µm (Figure 3.1 and Figure 3.2, respectively). Dynamic light scattering (DLS) is a valuable technique to measure the size, aggregation, and electrophoretic mobility of NPs by determining the Brownian movement of the NPs which are suspended in a liquid solution, while electro kinetic properties of the particles, such as surface charge, are measured jointly with laser Doppler velocimetry that measures the direction and speed of movement (Pelley and Tufenkji 2008). The DLS results reported from the current study expressed that CuO particles were aggregated in the exposure medium (seawater); the NPs showed larger aggregations than the MPs, and the aggregation level was displayed in a concentration response manner (Figure 3.3). Meanwhile, the zeta potential of both particles remained negative at all concentrations for both forms of particulate CuO, but at relatively low absolute values, indicating a propensity for aggregation of both CuO particulates in the exposure medium (seawater), but evidencing low stability (Table 3.1).

The findings detailed above are supported by a previous study by Griffitt et al. (2007), which reported that CuO NPs tend to aggregate in moderately hard freshwater, which influenced the level of the CuO NPs toxicity to the test species zebrafish (*Danio rerio*). Furthermore, the zeta potential of low negative value (-0.69mV) in the same study (Griffitt et al., 2007). Ahmed et al. (2010) reported that the majority of the CuO NPs dispersed in deionised water (DIW) appeared spherical in shape under TEM, while the DLS results showed the average hydrodynamic diameter and zeta potential to be 65.59nm and -39.67mV, respectively (Ahmed et al., 2010).

Similarly, Gomes et al. (2011) reported that CuO NPs dispersed in Milli-Q water were spherical in shape, with a mean diameter of 31 ±10 nm (TEM), and aggregate sizes ranging from 238 to 338nm (DLS) at 10µgL^{-1} (Gomes et al., 2011 and 2013). In addition, many recent studies using CuO NPs have used TEM to assess the shape and size of the particles. These authors reported that CuO NPs were spherical in shape, and that size was less than 50nm (17-45nm, 40nm and 22nm, respectively) (Dasari et al., 2013; Khan et al., 2013; Siddiqui et al., 2013). Moreover, the zeta potential of CuO NPs in distilled water and cell culture medium was highly negative (-20 and -25mV, respectively) (Siddiqui et al., 2013).

Buffet et al. (2011) reported that the size of CuO NPs increases dramatically when seawater is added to DIW, suggesting that seawater can influence the aggregation size of particles due to the specific chemistry and the presence of other particles, such as dust (Buffet et al., 2011). Similarly, Bondarenko et al., (2012) also claimed that the high hydrodynamic diameter of CuO NPs suspended in DIW could be due to partial NPs agglomeration due to the mineral salts (size ranged from 80 to 400nm). In addition, Buffet et al. (2011), determined that the type of aqueous media and high levels of instability of Cu ions can impact the zeta potential of CuO NPs and therefore aggregation level (positively charged (26.3 mV)) in DIW, while the NPs were found to be negatively charged in seawater medium collected at t = 0 and t = 2 days (-8.69 and -7.72mV, respectively). This large difference could be due to the reduction of electric repulsion forces and energy barrier between nanoparticles and the high salinity of seawater (Buffet et al., 2011; Khan et al, 2013).

The current findings as well as other findings mentioned above confirmed that the shape of CuO NPs (less than 50nm) was identical (spherical objects) to other studies using the same particle source (Sigma Aldrich), and the same visualisation technique (TEM), which also confirmed that different type of media used (seawater, Milli Q water or standard fresh water) had no effect on the shape of the particles. In terms of the potential effect of different particles' shape on exposed species, a study carried out by Piret et al. (2012) used two types of CuO NPs with similar chemical composition and size distribution, but different specific surface areas and different shapes (rod or spherical particles), to assess the potential effects on human hepatocarcinoma (HepG2). The results indicated a higher level of cytotoxicity, inflammatory and antioxidative

responses, and activation of intracellular transduction pathways, induced by the rod shaped CuO NPs on HepG2 cells compared to spherical CuO NPs at least at the higher concentrations (80mgmL^{-1}). The conclusion of this study revealed different toxicity of both CuO NPs could be explained by different specific surface areas, different shapes and the release of different amounts of Cu^{2+} (Piret et al., 2012)

Agglomeration of the NPs and their stability in various aqueous media are considered as major challenges in nanotoxicology, since these particles behave differently in different aqueous suspensions (Ahmed et al., 2012; Akhtar et al., 2012). In the current study, seawater tended to increase the aggregation of the particles. It has been reported that the size of CuO NPs increases dramatically when seawater is added to DIW suspensions, suggesting that seawater can influence the aggregation of particles, due to their chemistry and presence of other particles, such as dust (Buffet et al., 2011). Similarly, Siddiqui et al. (2013) also demonstrated that the particles size of CuO NPs in the aqueous medium increased to approximately five to ten times of the primary size of these particles.

The particle size of CuO particulates measured by DLS in the present study was larger than the particle size obtained by TEM and that might be due to the tendency of particles to agglomerate when suspended in aqueous media. The current finding was in agreement with numerous studies which reported that the aggregation of CuO NPs in aqueous media was common which led to the increase in particles size as measured by DLS (Sharma et al., 2009; Bai et al., 2009). Furthermore, there are other factors that may contribute to this difference in particles size as measured by TEM and DLS and that may due to the fact that different size determination techniques produce different results according to the principles employed. In fact DLS measures Brownian motion and subsequent size distribution of an ensemble collection of NPs suspended in liquid medium, which provides a mean hydrodynamic diameter often larger than the TEM diameter because it represents a dried layer of NPs on a TEM grid (Siddiqui et al., 2013). DLS also usually measures the size of clustered particles rather than individual particles (which is often the outcome of TEM measurements) (Sharma et al., 2012).

In addition, there is a tendency for particles (nano and micro) to aggregate in aqueous media due to several factors, including the ionic strength; and pH value of the media (Khan et al., 2013). Furthermore, since seawater was the medium of exposure in the current study, there are also other particles present in the seawater, such as dust and salt, which can also contribute significantly to an increase in the aggregation level of these copper particles (clusters) and therefore their size in the medium. This finding was supported by other investigators who used seawater as an exposure medium for CuO NPs (Gomes et al., 2011, 2012 and 2014).

4.1.1.1 The Limitations of NPs characterisation approaches

It is well known that NPs have unique physical and chemical features that are totally different from other larger particles of the same composition, and this makes these particles attractive to industrial and technology sectors to explore and use them heavily and properly in the near future (Peralta-Videa et al., 2011). Different techniques are available for characterising the properties of NPs, such as spectroscopy, microscopy, chromatography, filtration and centrifugation (Farré et al., 2009). Despite the power and the effectiveness of these techniques, there are several limitations and challenges. For example, they are destructive techniques, and therefore the sample can be analysed only once (Farré et al., 2009). Another major limitation is that the techniques operate in vacuum, which therefore measure only dry and solid samples (Farré et al., 2009).

Some of these techniques were successfully used in this study for measuring the size, shape, aggregation and zeta potential of CuO particles. However, this quantification cannot be achieved with these approaches alone; they can only provide an assessment of the fate of the NPs, which can contribute to the development of selective and sensitive analytical methods to be used in complex biological and aquatic matrixes (Farré et al., 2009). Despite the emerging research on the fate of NPs in the environment, there is an urgent requirement for fundamental information regarding releases, transformation, persistence, distribution and bioavailability of NPs in complex media (Sahu and Casciano 2009). Moreover, the development of the techniques requirement for the characterisation of NPs in environmental media is strongly required in order to gain a better understanding of these particles and their properties, in relation to their ecotoxicology to living organisms.

4.1.2 Copper concentrations

The potential uptake and accumulation of NPs in aquatic animals is of concern and only limited data regarding internal exposure concentrations and accumulation of NPs exist (Moore et al., 2006; Ward and Kach 2009; Gomes et al., 2011). In addition, the uptake, accumulation and related toxicity of NPs in aquatic organisms are dependent on several physicochemical features such as particle size, surface charge, shape, structure, particle chemistry and solubility and aggregation state (Bhatt and Tripathi 2011; Gomes et al., 2012). The uptake of NPs into aquatic organisms may occur via several routes of entry such as direct passage across gill and hepatopancreas (digestive or midgut gland, olfactory organs or body wall and other external surface epithelia) (Moore et al., 2006). Most NPs will enter into the cell via endocytosis, and the endocytotic pathways into cells which can either lead to the endosomal and lysosomal compartments (conventional endocytosis) or else via cell-surface lipid raft associated domains known as caveolae (Moore et al., 2006). In particular, the hepatopancreas in the invertebrate species is involved in uptake and digestion of food and storage of nutrient reserves, and is also responsible for intracellular lysosomal digestion of food via internalisation by endocytosis (Moore et al., 2004).

In the current study, the exposure of *M. edulis* and *M. modiolus* to different nominal concentrations of all three forms of Cu (nano, micro and salt) resulted in Cu being available to mussels regardless of the form used. However, only relatively low copper concentrations were observed in both mussel species flesh following exposure to particulate forms of CuO compared to the Cu salt. This was particularly evident in the *M. edulis* experiments (Figure 3.4 and Figure 3.10). Furthermore, the results from the presence of copper in the medium across the different treatments and concentrations did not show a clear pattern (Figure 3.4A and Figure 3.10A). It is possible that this was due to the dissolution of the CuO particles at these concentrations and during the timespan of these experiments (although this was not fully tested in this study). In addition, it is also possible that some loss of Cu has taken place due to adherence to the exposure tank walls. These results indicate that both mussel species, *M. edulis* and *M. modiolus*, accumulated more copper from the salt form ($CuSO_4$) suggesting a higher bioavailability from this form, compared to the particle forms (Figure 3.4B and Figure 3.10B). Numerous studies have shown the ability of Cu to accumulate in bivalves' tissues such as gills, digestive glands and gut (e.g. Regoli and Principato

(1995), Bebianno et al. (2004) and Serafim and Bebianno (2009)). However, data on the accumulation of Cu from Cu particles exposure are still sparse (Gomes et al., 2011; Canesi et al., 2012).

Studies assessing the accumulation of copper in mussel species from different forms reported different results. Gomes et al. (2012 and 2013) demonstrated that *Mytilus galloprovincialis* accumulated more Cu in the gills and digestive gland tissues from soluble Cu than CuO NPs during the first week of exposure to 10µgL^{-1} in seawater, and then accumulated more copper from CuO NPs that copper salt at the end of the exposure period (15 days). These studies concluded that copper salt showed more bioavailability and therefore was taken up at higher levels by the mussels when compared with CuO NPs (Gomes et al., 2012 and 2013). On the other hand, an opposite observation was reported by Golobič et al. (2012), who concluded that similar patterns of assimilated and depurated amounts of Cu and Cu body distribution were observed regardless of whether the exposed animal (terrestrial isopods, *Porcellio scaber*) was fed with Cu NPs or Cu salt. Furthermore, Gomes et al. (2012 and 2013) indicated that the longer period of exposure resulted in an increase in the accumulation of copper from CuO NPs into the mussels and that could be one of the main reasons behind the low accumulation of Cu from CuO NPs into *M. edulis* and *M. modiolus* in the current study (only 72 hours exposure period).

Additionally, there may also be other reasons for the low level of copper accumulated by the mussels after exposure to different concentrations of CuO particles (both NPs and MPs). Copper may be taken up in the dissolved form or directly as particulate matter. Both CuO particles showed high level of aggregation in the exposure medium (seawater), which may then be rejected back as pseudofaeces (Alshaeri et al., 2013). Some of the aggregated particles may break down into smaller particles, and then taken up and transferred into the haemolymph and circulating haemocytes (Moore et al., 2009; Gomes et al., 2011; Canesi et al., 2012). Additionally, aggregated particles may sink down and sediment to the bottom of the exposure tank by the time the experiment concludes, or stick to the wall of the exposure tank, reducing exposure and so not taken up by the mussels. Finally, Cu could have been eliminated during the sample preparation process, particularly the filtration step, during the Cu concentration analysis. In the current study, two filtration steps which were conducted during the sample

preparation process for Cu analysis, after HNO_3 digestion, to separate undigested tissues, and before using ICPOES, to avoid blocking the pipe during the analysis process. These filtration processes may influence the amount of Cu in the samples, at least to some extent.

The high accumulation of both CuO particles compared to the salt form ($CuSO_4$) cannot be just explained by the related metal accumulation in exposed mussels' tissues. This can be due to different accumulation trends that reflect distinct physiological and metabolic functions of tissues that when measured in the whole tissues are not able to discriminate tissue responses (Gomes et al., 2012). Overall, the uptake and accumulation of CuO NPs in mussel species varies and can be influenced by several factors such as different routes of exposure and uptake, aggregation state, the exposure media, exposure time, NPs concentrations, different behaviour in the experimental medium, different detoxification mechanisms and other physico-chemical properties mentioned above (Gomes et al., 2012). Further investigation needs to be carried out in order to develop an approach with improved processes for detection and quantification of the uptake of NPs, mode of action, and distribution inside tissues, cells and sub-cellular components of the organisms (Gomes et al., 2013).

As mentioned earlier, few data on the accumulation of Cu from Cu particles exposure are available in contrast to the extensive literature related to the accumulation of Cu from salt or ionic form. Therefore, several limitations exist in regards to the measurement of Cu released from these particles, including the lack of availability of precise techniques and methods to analyse Cu or other metals released from NPs, since the current techniques cannot differentiate between nanoparticulate and soluble metals (Griffitt et al., 2009). In addition, some chemical analysis techniques may involve a separation or filtration stage (especially tissue samples) in order to make them suitable for analysis, which could exclude a high amount of Cu or other metals and therefore would not detect the correct amount of Cu accumulated by the exposed organisms, as illustrated in the current results for Cu concentrations in the mussels tissues obtained by ICPOES.

4.2 Cytotoxicity

4.2.1 Cell viability

Cell viability is one of the most important biomarkers in wide use in many cytotoxicity studies. This is because assessing cell viability is an approach by which cell health can be assessed before subsequent studies. Assessment of cell viability can be performed using a wide range of techniques that are designed for use with selected cells, such as neutral red (NR) assay, and crystal violet (CV) stain, which are primarily used for lysosomes and live cell membranes; propidium iodide (Pi) dye for detecting dead cells using flow cytometry (FC) and trypan blue dye, which involves the use of a light microscope and is based on understanding of the exclusion of certain dyes by live cell membranes; and tetrazolium bromide (MTT) dyes, used to assess the mitochondrial dehydrogenases activity in cells (Jones and Senft, 1985; Pulskamp et al., 2007).

Two common techniques were chosen in the current study, to assess the cell viability of haemocytes of *M. edulis* and *M. modiolus*, trypan blue and flow cytometry (FC). This selection was based on several advantages associated with these techniques such as less cost, simplicity of use, low cell suspension required, reliable results and availability at the lab. In particular, flow cytometry (FC) offers the ability to count 50.000 cells per second, greatly increasing the reliability of the results (Diaz et al., 2010).

In the current study, all forms of Cu (nano, micro and salt) were confirmed to be toxic to both mussel species (*M. edulis* and *M. modiolus)* as indicated by the decrease in haemocytes viability when exposed to all forms of Cu for 72 hours at nominal concentrations of 5, 10, 15 and 20μgL^{-1}, and this decrease in cell viability was concentration dependent (Table 3.2: Table 3.3 and Table 3.4; Table 3.5). Furthermore, there was significant differences were observed for all form of Cu for both mussels. There was no significant difference between the results obtained from both techniques trypan blue and flow cytometry (FC) (Table 3.6).

Several studies have investigated the cytotoxicity of CuO NPs and MPs; however, results can vary and are influenced by many factors, such as cell types, exposure period, NP administration conditions, method selected and test sensitivity (Chibber et al., 2013). Chen et al. (2006) indicated that the nano form of Cu (23.5nm) reduced mice kidney cell viability significantly more than the micro form of Cu (17µm) after exposing cells to different concentrations of both forms of Cu for 72 hours. Similarly, Karlsson et al. (2008) demonstrated the cytotoxicity of CuO NPs and MPs (80µgmL $^{-1}$) on lung epithelial cells type II (A549) after 18 hours, and a reduction in cell viability, influenced by the concentration and exposure period and this cytotoxicity cannot by explained only by the release of Cu ions (Karlsson et al., 2008).

Another study used tetrazolium bromide (MTT) and neutral red uptake (NRU) assays to measure the cytotoxicity of CuO NPs in human pulmonary epithelial cells (A549) reported that CuO NPs significantly decrease cell viability in a dose-dependent manner (75% 66%, and 48%; 85%, 69% and 46%, respectively) after 24 hours of exposure to 10, 25 and 50µgmL^{-1} of CuO NPs, respectively (Ahamed et al., 2010). Furthermore, another study indicated that exposure of human pulmonary epithelial cells (A549) to 0.5 and 1mgL^{-1} of CuO NPs resulted in no significant cytotoxicity; however, a reduction in cell viability was observed 12 hours after increasing the concentrations of CuO NPs to 20 and 30mgL^{-1}, becoming greatest after 24 hours (Wang et al., 2012).

The release of Cu ions can be one of the main key factors behind cytotoxicity in all forms of Cu, as previously described. CuO NPs showed a higher reduction in cell viability than in other forms of Cu; especially the Cu salt (Karlsson et al. 2009). Similar cytotoxicity studies confirmed that CuO NPs resulted in greater cytotoxicity to exposed cells compared to larger particles from the same material composition, probably due to the large surface area associated with the smaller sized particles (Fahmy and Cormier 2009; Dey et al., 2012; Chibber et al., 2013). In contrast, other study concluded that Cu ions released from CuO NPs were not involved in the toxicity induced by these NPs on HepG2 cells after exposure to different concentrations of CuO NPs (0, 2, 5, 10, 25 and 50 mgmL^{-1}) for 24 hours (Siddiqui et al., 2013). Many studies reported that there was an efficient NP internalization at early exposure time even at mitochondrial level, which then led to progressive worsening of mitochondrial morphological functional conditions as well as generalised ultrastructural damage that caused cell death by necrotic changes

(Karlsson et al. 2008; Lanone et al. 2009). The Cu ions contributed significantly to mitochondria damage in the organisms tested and the possibility that other toxicity mechanisms or pathways may be involved is high (Sun et al. (2012).

In addition, another explanation for the high cytotoxicity of CuO NPs is that exposure to these particles might generate reactive oxygen species (ROS) and damage cell mitochondria, and therefore lead to cell death. This finding was also suggested by Chibber et al. (2013) indicating a possible involvement of oxidative imbalance in the first step of cytotoxic events triggered by CuO NPs (Chibber et al., 2013). Similarly, Fahmy and Cormier (2009) indicated that the cytotoxicity induced by CuO NPs was mediated by the generation of oxidative stress in these cells and only CuO NPs among other metal oxide NPs were able to inhibit effectively the activity of catalase and GR enzymes and increase the activity of GPx as compared to cells exposed only to medium (Fahmy and Cormier 2009).

The high cytotoxic effects of CuO nano particles shown above are concentration dependent. This is probably driven by multiple factors, including the large surface area relative to the small size, which allows more Cu particles to interact with the organisms and higher reactivity. Other physiochemical properties are also relevant; including metal content, aggregation state and charge, and factors mentioned previously such as oxidative stress (after exposure to CuO NPs caused by ROS generation), release of Cu ions into exposed media (Xie et al., 2011; Buffet et al., 2012; Chibber et al., 2013; Siddiqui et al., 2013).

As mentioned above, both assays were utilised in this study (flow cytometry and trypan blue assays). Each returned similar results for the cell viability of both types of mussels. Both techniques were applicable for assessing cell viability in the conducted study, and were also suitable for testing the haemocytes of both mussels (*M. edulis M. modiolus*). A statistical comparison indicated no significant difference between the results obtained from both assays. However, flow cytometry (FC) is preferable, and recommended, because it offers several advantages, including that it is less time consuming and can obtain results with greater reliability (100.000 cells per second).

4.3 Genotoxicity

4.3.1 DNA damage

The comet assay technique was chosen in this study as it is widely accepted and used in many laboratories and field studies, because of its applicability to a wide range of different cells (for example, fish, mussels and human cells) and its many other benefits. For example, ease of use, speed, simplicity, low cost, flexibility, sensitivity enabling detection of small amounts of DNA damage, the small number of cells required for the experiment (50-100 per sample), and the delivery of reliable results (See 1st chapter). Despite the benefits of using the Comet assay, this technique may become complicated and take longer to obtain results where there are a larger number of samples (more than 50 samples) (Woods et al., 1999; Tice et al., 2000; Singh and Hartl 2012).

In the present study, DNA damage was measured using the Comet assay after an exposure of haemocytes and gill cells of *M. edulis* and *M. modiolus* to different concentrations (5, 10, 15 and 20µgL^{-1}) of the three forms of Cu for 72 hours. Clearly, the results showed that all forms of Cu tested had the potential to cause DNA damage in a concentration dependent manner to both cells of both mussels (although damage was more pronounced in gill cells than haemolymph cells) even at low concentration (5µgL^{-1}) (Figure 3.6; Figure 3.7 and Figure 3.11, respectively). The majority of cells in the control treatment showed minimal and low grade of damage (less than 6%), and this is an acceptable background level arising from either natural or manmade stresses in the field or during the processes of the comet assay.

A comparison of all forms of Cu tested in the present study indicated that the CuO NPs were the most toxic form to exposed cells (23% and 26%; 20% and 22% in DNA tail of haemocytes and gill cells of *M. edulis* and *M. modiolus*, respectively at 20µgL^{-1} after exposure to CuO NPs). In contrast, less DNA damage was measured in cells exposed to MPs and the salt form at the same equivalent concentration (19% and 21% and 14% and 16% in DNA tail of *M. edulis* and 17% and 18%; 14% and 14% in DNA tail, respectively) (Figure 3.7 and Figure 3.11, respectively). These findings were consistent with the cell viability results, reflecting the same pattern of toxicity of all forms of Cu.

The findings presented here for CuO NPs are in agreement with previous studies used to investigate the genotoxicity of CuO NPs. For example, Karlsson et al. (2008 and 2009), compared the toxicity of multiple metal oxide particles (including CuO) on human cells (human pulmonary epithelial cells (A549), and reported that CuO NPs caused significant DNA damage when cells were exposed to $40\mu gL^{-1}$ for 4 hours (41% tail DNA), compared to other metals (TiO_2, ZnO, $CuZnFe_2O_4$, Fe_3O_4, Fe_2O_3 and MWCNT). These studies suggested the high toxicity of CuO NPs may result from the fact that Cu is a transition metal, causing oxidative stress via the Fenton reaction (Karlsson et al. 2008 and 2009). Similarly, a study was conducted to measure DNA damage in human pulmonary epithelial cells (A549) exposed to $50\mu gmL^{-1}$ of CuO NPs for 24 hours, using Western blot analysis. This demonstrated that DNA damage was higher in treated cells than in untreated cells, and suggested that oxidative stress and lipid peroxidation contribute to the high level of DNA damage in cells treated by CuO NPs (Ahamed et al., 2010). Bondarenko et al. (2012) subsequently observed an increase in ROS production after 4 hours and DNA damage after 8 hours in bacteria (*E. coli*) exposed to $0.1mgL^{-1}$ of CuO NPs; they hypothesised that Cu ions released from CuO NPs and ROS generation triggered the observed DNA damage response.

Moreover, CuO NPs have also been shown to create considerable DNA damage in a time-dependent manner in algae and human cells exposed to 0.5 and $15mgL^{-1}$, for 72 and 48 hours, respectively (Wang et al. 2011 and 2012). The conclusion of the latter studies was that DNA damage is detected after ROS formation, which could be an outcome of the ROS generation mechanism and therefore oxidative stress; however, the interaction between ROS and DNA damage needs further investigation to confirm this finding (Wang et al. 2011 and 2012). Another interesting study (Gomes et al., 2013) investigating DNA damage in the haemocytes of *Mytilus galloprovincialis* after exposure to $10\mu gL^{-1}$ of CuO NPs for 15 days, reported that DNA damage increased with time in exposed mussels, and was greater than in unexposed mussels. The authors concluded that the formation of ROS could explain the major effects of CuO NPs in mussel haemocytes (Gomes et al., 2013).

The potential for direct and indirect DNA damage is caused by several external factors, such as exposure to particles with particular unique physical and chemical properties, including small size, aggregation, high reactivity and large surface area, as well as ionic transition metals released from the particles (for example Ag, Cu and Zn). These contribute to the production of ROS, and therefore oxidative stress (Gomes et al., 2013). Many possible factors could be behind the DNA damage in tested cells of both mussels. The production of ROS may be the key factor for damaging the DNA in cells exposed to Cu forms. Ahmed et al. (2010) demonstrated that DNA damage was higher in the cell exposed to CuO NPs for 24 hours, indicted by the increase in the expression level of Hsp70 and p53 protein in treated cells. In addition, Wang and colleagues determined the relationship between ROS generation and DNA damage and concluded that oxidative stress was the primary toxic effect (Wang et al., 2012). In addition, cellular internalisation of NPs can induce an interaction with DNA inside the nucleus, or even during mitosis, where it can induce various forms of DNA damage (Handy et al., 2008; Gomes et al., 2013).

In addition, there were also issues concerning the propensity of NPs to aggregate in multiple aquatic media (e.g. DIW, Milli Q water and seawater). This aggregation of NPs may contribute to the toxicity of these particles, heightening oxidative stress and DNA damage. In the present study, CuO NPs were found to be highly aggregated in the exposure medium (seawater), and the particles uptake by the digestive system was dependent on particle size and other particle properties. The aggregated particles may break down into smaller particles, be accumulated and transferred into the haemolymph and circulating haemocytes according to previous studies (Moore et al., 2009; Gomes et al., 2011; Canesi et al., 2012). Furthermore, exposure time was a key factor increasing NPs aggregation and accumulation. However, additional investigation is needed to confirm the impact of the exposure period on the level of NP toxicity (Gomes et al., 2013).

Another possible factor that may contribute to DNA damage is the accumulation of Cu in the mussels' tissues at least to some degree. In the current study, no clear pattern was observed in the accumulation of Cu for any of the forms. However, both mussels accumulated more Cu from the salt form ($CuSO_4$) than from the particles. Based on this result, the genotoxicity of the different Cu forms was not related to the level of Cu

accumulation in the mussels' tissues. Gomes et al. (2013) supported this view latter argument that if the Cu accumulation in the *M. galloprovincialis* tissues explained the effects observed, then the genotoxicity of both forms of Cu (nano and salt) should be the same (since the same mass was added). This study concluded that the level of NPs accumulation in mussels' tissues may not be directly related to cellular DNA damage (Gomes et al., 2013). This is because mussels are a filter feeding species, filtering water through their gills to capture food such as plankton and algae, including NPs, but are also able to reject unwanted particles via pseudofaeces formation (Moore 2006; Handy et al., 2008; Canesi et al., 2012). It has been reported that copious amounts of pseudofaecal material were expelled by the exhalant siphon of the *M. edulis* when fed with *Tetraselmis suecica* algae that had taken up previously SWCNTs, and SWCNTs were clearly seen to be discarded by the mussels bound up in pseudofaeces (Alshaeri et al., 2013).

Moreover, the accumulation of NPs in mussel tissues (such as gills) may not be the same for multiple NPs (including CuO NPs, Ag NPs, and ZnO NPs). This difference could be due to various accumulation patterns that reflect the distinct physiological and metabolic functions of tissues. Currently, little information exists on the bioavailability of different NPs to aquatic organisms such as mussels, route of exposure and uptake mechanisms, as well as their ingestion rates, internal exposure concentrations and cell and tissue distribution. At the present, a major challenge is the development of approaches that allow accurate detection and quantification of the uptake of NPs, distribution inside tissues, cells and sub-cellular components of the organisms. Additionally, numerous constraints arise when using NPs, related not only to their size and quantity but also to inherent particle properties (Ward and Kach, 2009; Bhatt and Tripathi, 2011; Canesi et al., 2012).

4.3.2 Oxidative stress

Oxidative stress is a major mechanism by which NPs exhibit toxicity (Handy et al., 2008; Dhawan and Sharma 2010). Addressing the effect of oxidative stress in cells exposed to NPs can be achieved by assessing several antioxidant enzymes and levels of lipid peroxidation and DNA damage within the treated cells, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxide (GPX), as well as lipid peroxidation levels such as the activity of thiobarbituric acid-reactive substances (TBARS). In the current study, oxidative stress was evaluated in relation to mussel's gill cells, by assessing one of the antioxidant enzymes in the cells superoxide dismutase activity (SOD) (expressed as the induction of SOD inhibition) using a SOD assay kit, and lipid peroxidation (expressed by an increase in TBARS) using the TBARS assay.

The results of assessing SOD activity and TBARS levels in the gill cells of both mussel species revealed a rapid significant increase in SOD activity (which was expressed as a reduction in SOD inhibition) and TBARS activity, which indicated oxidative stress and lipid peroxidation after exposure to different concentrations of Cu forms (Figure 3.8; Figure 3.9 and Figure 3.12; Figure 3.13, respectively). In addition, the toxicity of CuO NPs was compared to other forms, indicating that SOD activity and lipid peroxidation were significantly higher in those *M. edulis* exposed to CuO NPs than in mussel cells exposed to other forms of, which correlates well with the results reporting cell viability and DNA damage (Figure 3.8 and Figure 3.9). However, there was no significant difference in SOD activity observed in gill cells of *M. modiolus* exposed to all forms of Cu, while, lipid peroxidation was significantly higher in the gill cells of *M. modiolus* exposed to CuO NPs compared to other forms of Cu; this was in agreement with the lipid peroxidation results in *M. edulis* (Figure 3.12; Figure 3.13).

Oxidative stress and lipid peroxidation are known to be essential mechanisms of cell damage, and occur in response to several types of metal and metal oxide NPs (Stone et al., 2007; Fahmy and Cormier 2009). The same genotoxic effects of CuO NPs were studied in several studies concerning ROS, leading to oxidative stress and lipid peroxidation. For example, Fahmy and Cormier (2009) demonstrated that CuO NPs caused oxidative stress in human laryngeal epithelial cells (HEp-2) in a dose-dependent manner, as reflected by the imbalance in several enzymes SOD, CAT, and GPX in

treated cells. They also proposed that an increase in antioxidant enzymes indicates the impairment of epithelial cells to scavenge ROS, as generated by CuO NPs, which may result in oxidative damage and subsequently cell death (Fahmy and Cormier 2009). However, mussel species have an ability to resist slight changes in environment conditions as well as other external stress such as pollution and NPs, and may recover since these species live relatively long periods of time; alternatively, lack of recovery may lead to cell dead and therefore mussels dead.

In a later study, Ahamed et al. (2010) illustrated that CuO NPs potentially cause genotoxicity in human pulmonary epithelial cells (A549), as was indicated by an increase in oxidative stress and lipid peroxidation, which occurred in cells exposed to concentrations of CuO NPs in the range of 10–50µgmL^{-1}. The study observed that malondialdehyde (MDA) level, as an end product of lipid peroxidation and the activity of antioxidant enzymes CAT and SOD, was significantly higher in treated cells than in untreated cells, and so, concluded that oxidative stress may be the primary mechanism for the toxicity of CuO in human lung cells (Ahamed et al., 2010) .Analogously, Sun et al. (2011) also observed high ROS production in cardiac microvascular endothelial cells (HCMECs) after 12 and 24 hours of exposure to CuO NPs at concentrations of 0.001–100 µgmL^{-1}. They hypothesised that exposure time and concentrations of NPs account for only a small proportion of oxidative stress and lipid peroxidation (Sun et al., 2011).

Following this, Gomes et al. (2011 and 2012) suggested exposure to 10µgL^{-1} CuO NPs lasting for a long period of time (15 days), led to breakdown in antioxidant defence enzyme activities (including SOD, CAT AND GPX), as well as lipid peroxidation which caused oxidative stress in the gills of *Mytilus galloprovincialis* (Gomes et al., 2011 and 2012). Later, Siddiqui et al. (2013) conducted a similar study examining the possible mechanisms resulting in oxidative stress and lipid peroxidation, by exposing human hepatocellular carcinoma cells (HepG2) to CuO NPs (22 nm) in a concentration range from 2–10mgmL^{-1}. The results showed CuO NPs induced intracellular production of ROS in a dose dependent manner, and that lipid peroxidation (expressed as the increase in MDA level) was significantly higher in exposed cells. This indicated that particle size and the large surface area of CuO NPs contributes to generating ROS and increasing lipid peroxidation (Siddiqui et al., 2013). Clearly the particle size of CuO

NPs play an essential role in the toxicity of CuO NPs and influence other properties such as aggregation and surface charge, as illustrated in this study.

Analogously, Ivast et al. (2010) and Dasari et al. (2013) reported that CuO NPs generated higher levels of ROS in the bacterium *E. coli* than other multiple metal oxide NPs (TiO_2, ZnO NPs). Aruoja et al. (2009) indicated that CuO NPs were more soluble and more toxic than bulk CuO to algae *P. subcapitata* (CuO: 100mgL^{-1}). However, in the present study, no significant different was observed between the toxicity of all forms of Cu in terms of SOD activity in *M. modiolus* cells, which could be due to the difference in species used and cell types. A further comparative study was carried out by Mortimer et al. (2010) to measure the toxicity of CuO NPs and MPs on protozoa (*Tetrahymena thermophile*). Results suggested that CuO NPs induced a generation of ROS that represented approximately 10–20 times higher toxicity than CuO MPs (Mortimer et al., 2010). Recently, Perreault et al. (2012) investigated the genotoxic effects of CuO NPs after exposure of N2A mouse neuroblastoma cell lines for 24 hours. They demonstrated oxidative stress in exposed cells expressed as a formula of MDA from CuO NPs concentrations of 25mgL^{-1} and higher (Perreault et al., 2012).

The present results on mussel species are in agreement with the results reported above from other studies which demonstrated that the generation of ROS is one of the main mechanisms for the toxicity of CuO NPs, as reflected by oxidative stress and lipid peroxidation. One of the possible factors leading to oxidative stress and lipid peroxidation is the release of Cu ions from the surface of CuO NPs when introduced into an aqueous media (Karlsson et al., 2008; Ahamed et al., 2010) .Heinlaan et al. (2008) argued that the toxicity of CuO NPs on bacteria *V. fischeri* and crustaceans *D. magna* and *T. platyurus* could be largely explained by the presence of soluble Cu ions. Further, Karlsson et al., (2008 and 2009) concurred that the release of Cu ions from NPs, which are toxic to exposed cells, is a possible factor resulting in oxidative stress in human cells (Karlsson et al., 2008 and 2009).

Conversely, Fahmy and Cormier (2009) claimed that the release of Cu ions from NPs did not contribute to either cytotoxic response or oxidative damage (Fahmy and Cormier 2009). Another study supported the latter argument, demonstrating the amount of Cu ions released did not contribute to oxidative stress or mortality in zebrafish exposed to CuO (Griffitt et al., 2007). These two studies hypothesised that oxidative stress, lipid

peroxidation and the decrease in mortality associated with exposure to CuO NPs were generated by the particle itself (their properties such small size and large surface area, contributed to this effect), rather than released Cu ions (Griffitt et al., 2007; Fahmy and Cormier 2009).

According to the results obtained in the present study, there are two main factors contributed to the generation of ROS, which caused oxidative stress and lipid peroxidation, indicated by an increase in SOD activity and TBARS activity in mussel cells exposed to all forms of Cu. Firstly, the release of Cu ions for all Cu forms contribute to at least small proportion of Cu NPs toxicity on gill cells of *M. edulis* and *M. modiolus* as illustrated in the results, due to the catalytic effects of this transition metal. However, further research is required to improve and develop new techniques and approaches for assessing the Cu release from individual particles metal in order to gain better understanding of the ability of Cu ions to contribute to Cu toxicity. Secondly, the unique properties of CuO NPs, including their small size, large surface area and copper accumulation in the mussel species. The particle size of CuO NPs play an essential role in the toxicity of CuO NPs and influence other properties such as aggregation level and surface charge, as illustrated in this study. Furthermore, it has been reported that particle size and the large surface area of CuO NPs contribute to generating ROS and increasing lipid peroxidation (Siddiqui et al., 2013).

An additional potential factor causing oxidative stress and lipid peroxidation when exposed to CuO NPs is exposure time. It has been reported that long periods of exposure to CuO NPs increase the aggregation of particles in the media and generate relatively high ROS, which then cause oxidative stress and lipid peroxidation (Gomes et al., 2012). In this study, the exposure period was only three days (72 hours), which was enough for observing the oxidative stress in mussels treated with all forms of Cu. In addition, based on these results and the results reported in the studies mentioned above, the test organism and cell types are additional factors leading to increased toxicity of CuO NPs. Perreault et al. (2012) indicated that the lower sensitivity of N2A cells can result from different agglomeration states of CuO NPs in a culture medium, which may lead to oxidative stress.

4.4 Comparison toxicity of the various forms of Cu

In the current study, three forms of Cu were chosen for use in each experiment (nano, micro and salt), in order to address the toxic effect of each form and to compare toxicity in terms of the selected endpoints (Cell viability, DNA damage and oxidative stress). A statistical comparison of the toxicity of all forms of Cu indicated that CuO NPs were the most toxic forms of Cu, causing cytotoxicity and decreasing cell viability, DNA damage and oxidative stress in both the haemocytes and gill cells of both mussels (*M. edulis* and *M. modiolus*) compared to micro and dissolved salt (apart from SOD activity in *M. modiolus* mussels).

The small size of the CuO NPs can play an important role in the genotoxicity of the NPs (including CuO NPs), by introducing them to biological systems, such as the human body. NPs can cross the biological barriers in the lung, gut, or brain, and the aggregation of NPs inside the biological system can alter biological responsiveness, due to a reduction in the surface charge area (Lövestam et al., 2010; Bhatt and Tripathi 2011). Moreover, the small size of the NPs influences other NPs properties, such as surface charge, aggregation level, reactivity and solubility. This may result in more toxic effects affecting the exposed organism (Oberdörster 2009; Dhawan and Sharma 2010). The majority of studies measuring CuO NPs toxicity have confirmed that the unique properties of CuO NPs seem to contribute to the toxicity of CuO NPs (Griffitt et al., 2007; Fahmy and Cormier 2009; Ahamed et al., 2010; Buffet et al., 2011; Gomes et al., 2011 and 2012).

The high toxicity of CuO NPs compared to that of larger particles of Cu may be explained by their large surface area because of their small size and the tendency of Cu NPs to aggregate in seawater. It is possible that the surface of the CuO has the ability to generate ROS such as H_2O_2 in the cell medium, which can then diffuse across the cell membrane of HEp-2 cells (Schubert and Wilmer 1991). According to Gomes et al. (2011), mussel gills filter the water from surrounding area and dissolved Cu released from the NPs can be taking up combined with a cellular uptake of nanoparticles aggregates. Moreover, CuO NPs can pass the cellular membrane, enter inside the cell, dissolve rapidly and release high concentrations of ions sufficient to disrupt Cu homeostasis and generate radicals and this NPs mechanism of toxicity known “Trojan

horse-type mechanism", which was identified in cell cultures (Moore 2006; Griffitt et al., 2009; Karlsson et al., 2008).

A study of the toxicity of nano and micro particles of CuO on human cells (lung epithelial cell line A549) reported that the nano form of CuO was markedly more cytotoxic and genotoxic than the micro particle form (Karlsson et al., 2008 and 2009). It concluded that the high toxicity of CuO NPs was driven by the high surface reactivity, because of the small size of the particles, and the release of Cu-ions that are toxic to the cells (Karlsson et al., 2008 and 2009). Chen et al. (2006) also reported that CuO NPs (23.5nm) were significantly more toxic than micrometer particles when orally ingested by mice, as a result of their small size.

Mortimer et al. (2011) suggested that the higher ability of CuO NPs to cause oxidative stress may be related to the changes in the fatty acid composition of their test species cells (*T. thermophile*). This study also indicated that the changes in lipid biosynthesis, including decrease in unsaturated fatty acids, increase stress resistance and longevity of *C. elegans* (Shmookler Reis et al., 2011). SOD activity has been shown to be significantly higher in animals (*S. plana* and *H. diversicolor*) exposed to CuO NP compared to animals exposed to soluble Cu of large particles, which leads to oxidative stress (Buffet et al., 2011). On the other hand, few studies observed no significant difference in the toxicity between different forms of Cu. For example, Buffet et al. (2012) reported that both forms of Cu (nano and salt) were able to generate ROS and therefore oxidative stress in tested species (*S. plana,* and *H. diversicolor*) however; no significant difference was detected between the two Cu treatments in both species after 21 days of exposure. Interestingly, Gomes et al. (2013) found out that ionic Cu showed higher genotoxic effects the nano form of CuO in *M. galloprovincialis* exposed for 15 days.

Another possibility is that the dissolution level of CuO NPs increases the cytotoxic and genotoxic effects from Cu NPs. Maria and Bebianno (2011) reported that levels of free Cu ions can determine the different modes of action of the mussel's antioxidant system. Perreault et al. (2012) showed that CuO NPs agglomerates in the culture medium and genotoxic effects were observed at lower concentrations than lipid peroxidation induced by oxidative stress. This may indicate a different mode of action for small compared to larger CuO NP agglomerates and this effect may be due to a more important

contribution of the soluble copper fraction under our conditions (Perreault et al. 2012). Bondarenko et al. (2012) also hypothesised that Cu ions solubilised from CuO particles triggering the generation of ROS and DNA damage response in bacteria cells *E. coli*.

Furthermore, recent ecotoxicological studies have reported toxicity following exposure to Cu salt and CuO NPs (Griffitt et al., 2007; Heinlaan et al., 2008). The toxicity of CuO NPs was much higher than the CuO MPs, due to the solubilised bioavailable fraction of the copper (Heinlaan et al., 2008). The results of Shi et al. (2011) indicated that CuO NPs decrease chlorophyll content of the duckweed and that CuO NPs toxicity is three to four times higher than that of ionic Cu, due to high level of Cu uptake from NPs. Additionally, Mortimer et al. (2011) reported the opposite, and concluded that the toxicity of CuO NPs to *T. thermophile* cannot only be explained by the Cu ions dissolved from NPs, but rather it displays a distinctive toxicity pattern (Mortimer, et al., 2011).

Overall, the nano form of Cu appears to be more toxic to the cells of both mussel species, more so than micro and salt forms. These effects are primarily influenced by the special features of NPs, including their small size, large surface area and high aggregation, which affect the amount of Cu ions released into the exposure media and inside the exposed cells. However, further investigation is required to gain a fuller understanding of the potential mechanisms of CuO NPs toxicity, and the possible factors that contribute positively to their toxicity. Additionally, The contribution of particle solubilisation, both in the media and inside the cell (Trojan Horse effect), requires more investigation in the near future in order to provide a better knowledge of the genotoxic potentials of CuO NPs at the cellular level.

4.5 Comparison between *M. edulis* and *M. modiolus*

The benefits of using aquatic invertebrates as test organisms for assessing pollution, including from NPs, are extremely large, since invertebrates represent about 95% of animal species, have an important ecological role and represent potential food sources for many animals, including humans (Baun et al., 2008). In particular, bivalves' species such as mussels have been used heavily in many pollution assessment studies and a common example for pollution monitoring called "the Mussel Watch". The Mussel Watch concept was introduced in 1976 in a surveillance of U.S. coastal regions. This includes different species of mussels (*Mytilus*) and oysters (*Ostrea* or *Crassostrea*) as surveillance organisms for assessing pollutant levels and their changes with time in many coastal regions in US (Goldberg et al., 1978).

The results of the biomarkers tested in the present study showed that all Cu forms decrease cell viability (Table 3.6), increase DNA damage (Figure 3.14; Figure 3.15 and Figure 3.16), cause oxidative stress (expressed as a reduction in SOD inhibition) (Figure 3.17), and lipid peroxidation (expressed as an increase in TBARS activity) (Figure 3.18) in the exposed haemocytes and gill cells of both mussels (*M. edulis M. modiolus*). In particular, *M. edulis* cells were more sensitive to Cu forms than *M. modiolus* (gill cells were the most susceptible), especially when exposed to nano forms of CuO.

Available literature related to the genotoxicity of NPs in mussels species suggests oxidative stress is a key factor in DNA damage to mussel's haemocytes, because haemocytes, as components of the open circulatory system are vulnerable to NP uptake, which leads to toxicity (changes in immune parameters (enzymes and antimicrobial peptides) and ROS production)) (Canesi et al., 2012; Gomes et al., 2011 and 2013). In the current study, DNA damage in gill cells was clearly significantly higher than in the haemocytes of *M. edulis*; this significant DNA damage in gill cells may be due to the fact that gill tissues are in constant contact with the water column, which contains food and harmful particles, such as NPs.

Based on the results obtained in the current study, several factors can be seen influencing the level of toxicity in both mussel species (*M. edulis M. modiolus*). The first factor is the difference in the physiological mechanisms involved in the feeding process. The amount of NPs aggregates taken up into mussel via gills and then transferred directly to the digestive system, where disaggregation process occurs, is important aspects. After that, nanosized particles can be then potentially transferred from the digestive system to the haemolymph, and to circulating haemocytes (Canesi et al., 2012).

The second factor is the difference in the mussel species' habitats: they both experience different environmental conditions, such as temperature and tidal movement, and various levels of sunlight, which may contribute to the threat to the mussel species. *M. edulis* lives in a community in intertidal areas and in shallow waters, which are vulnerable to external threats from surrounding areas; such as pollutants in sewage water, discharge from vessels and coastal soil degradation. In contrast, *M. modiolus* mussels live in subtidal areas in large habitats with enormous diversity in depths of around 20m or more, where the exposure to man-made stressors is considered lower. The third factor that may have contributed to the ability of *M. modiolus* to apparently cope better with stress from NPs is their difference in size and life span. *M. edulis* has an average size of between 5 and 10cm, which may result in less adaptability to change in environmental conditions, such as external threats; it also lives around 24 years. *M. modiolus* is double the size of *M. edulis*, between 15 and 20cm. This is likely to increase their tolerance to environmental stressors. They also live longer, for approximately 45 years (Anwar et al, 1990). Moreover, mussel species are able to detect and respond to increased Cu concentration in their environment by an intermittent movement of the shell valves, along with periodic opening and closing of the exhalent siphon which called ``testing behaviour'' (Davenport and Manley 1978). In regards to the latter argument, Curtis et al. (2000) assessed valve and cardiac responses (the valve movements) in *Mytilus edulis* after exposed to Cu on a long-term basis. The results indicated that *M. edulis* started to respond to an increase in Cu concentrations (testing behaviour) at a copper concentration of 0.8µM and then the valves closed completely at copper concentrations of 3.1µM (remaining close for 3 hours). Therefore, because of this behaviour, mussels may remain close for a period of time, which then decreases the O_2 and increase the CO_2 levels in the mantle cavity-water, and therefore influence their

health. This conclusion supports the results obtained in the present study and indicates that due to the large size of *M. modiolus* mussels, they may be able to respond better to the copper exposures. In summary, the use of both mussel species (*M. edulis and M. modiolus*) was important to assess the wider toxicity of CuO forms (nano, micro and salt) in the marine environment, by focussing on two similar particle feeders with different habitats and ecology. In particular, *M. modiolus* results are important as populations of this species create important habitats that support highly diverse communities, and as such are an example of biogenic reefs under the Habitats Directive description of reefs (Mair et al., 2000). Hence, increasing our focus on this essential species, in particular by studying NP toxicity is significantly vital in order to understand any potential threats. Different mussel species and cell types may lead to different findings concerning NPs toxicity, as shown in this study. Finally, further research is needed using mussel species, to improve the understanding of the mechanisms of CuO NPs toxicity in regard to their uptake and accumulation, the importance of bioavailability, and particle aggregation over long periods.

5 CONCLUSION

Cu has been known as one of the toxic metals to living organisms at various levels and the toxicity of Cu had been extensively studied previously. Cu NPs have unique physical and chemical properties that are different from their larger counterpart such as small size, large surface area and aggregation state. These particular features allow Cu NPs (such as CuO NPs) to be an essential ingredient in many commercial products and applications such as gas sensors, batteries, pigments, air and liquid filtration materials and optical equipments (Ahamed et al., 2010, Dasari et al., 2013). As a result, these NPs will likely release into the environment, particularly the aquatic environmental, which is the ultimate sink for pollutants discharged from varies sources.

In the present study, both particle sizes of CuO (NPs and MPs) were prepared in seawater and observed under TEM. In addition, the DLS results indicated that both particles were aggregated in the exposure medium (seawater); the NPs had larger aggregates than the MPs and the aggregation level was dependent on the concentration. Moreover, all forms of Cu were found to be bioavailable to mussels regardless of the form used (NPs, MPs or salt), and only low copper levels were found in both mussel species on exposures to particulate CuO, compared to higher levels of copper in the salt form ($CuSO_4$) exposures at all concentrations.

Results obtained from this study showed that all three forms of Cu (nano, micro and salt) have the potential to decrease the cell viability in haemolymph cells for both mussels *M. edulis* and *M. modiolus* in a concentration response manner. Similarly, nano, micro and salt forms of Cu can cause DNA damage in both types of cells (haemolymph and gill) for *M. edulis* and *M. modiolus* mussels even at low concentrations ($5\mu gL^{-1}$) and the level of damage is dependent on the concentration of Cu. Moreover, SOD activity and lipid peroxidation were observed to have increased in the gill cells of each mussel exposed to all forms of copper, indicating increased oxidative stress.

In addition, this study indicated that CuO NPs were the most toxic form of Cu tested, causing cytotoxicity and decreasing cell viability, increasing DNA damage and oxidative stress in both the haemocytes and gill cells of both mussels (*M. edulis* and *M. modiolus*), compared to micro (CuO MPs) and dissolved salts (CuSO4) (apart from SOD results of *M. modiolus* mussels). The high cytotoxicity and genotoxicity of the

nanoform of Cu are primarily influenced by the special features of NPs, including their small size, large surface area and high aggregation in the medium, which affect the amount of Cu ions released into the exposure medium and inside the exposed cells.

The blue mussel *M. edulis* cells were more sensitive to Cu forms than the horse mussels *M. modiolus* (gill cells were the most susceptible), especially when exposed to the nano form of CuO. Many key factors can be behind the level of toxicity evidenced by both mussel species. Firstly, differences in the physiological mechanisms involved in the feeding process of both mussels, which may influence the particle uptake. Secondly, the difference in the mussel species' habitats: they both experience different environmental conditions, such as temperature and tidal movement, and various levels of sunlight, which may contribute to the threat to the mussel species. Finally, the difference in size and life span, *M. edulis* has an average size of between 5 and 10 cm, which may result in less adaptability to change in environmental conditions, such as external threats; it also lives around 24 years. *M. modiolus* is double the size of *M. edulis*, between 15 and 20 cm. This is likely to increase their tolerance to environmental stressors. They also live longer, for approximately 45 years (Anwar et al, 1990).

Overall, the level of CuO NPs toxicity on living organisms can be influenced by several factors such as the test species, cell types, concentrations, exposure media and time of exposure, as illustrated in the present study. As detailed in previous chapters, effect mechanisms relating to CuO NPs toxicity are still not fully understood, and understanding the underlying mechanisms relating to the potentially adverse effects of CuO NPs on mussels organisms is a prerequisite for determining appropriate test strategies. Furthermore, the use of both mussel species was important to assess the wider toxicity of CuO forms in the marine environment, by focussing on two similar particle feeders with different habitats and ecology. Different mussel species and cell types may lead to different findings concerning NPs toxicity, as shown in these results. Finally, further research is needed using mussel species, to improve the understanding of the mechanisms of CuO NPs toxicity in regard to the uptake and accumulation of CuO NPs, the importance of bioavailability, and particle aggregation over long periods.

6 FUTURE WORK

The environmental effects of nanoparticles (NPs) including (CuO NPs) are still of concern even with the current data and knowledge of the ecotoxicology of NPs, due to many reasons. The first reason is the vast expansion of using these particles in the commercial and industrial productions and persistency of many types of nanoparticles, and these materials by the time will likely accumulate in the environment. Secondly, the complexity of NPs in terms of their fate and behaviour in the environment make assessing their impacts hard to predict and confirm. Therefore, further study and research are needed in this field. My future work related to the current study will focus on the following:

- The fate and behaviour of CuO NPs in the marine environment need to be well understood as this will explain more their ecotoxicity.
- Further study regarding the physical and chemical characteristics of CuO NPs.
- CuO NPs transfer to primary target species such as mussels via potential food chain source such as algae.
- Assessing the influence of different exposure media on NPs CuO NPs ecotoxicity.
- Assessing the toxic effect of different size of CuO NPs on marine mussels, for better understanding of the particle size influence.

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